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RECOMBINANT INFECTIOUS LARYNGOTRACHEITIS VIRUS AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 08/126,597, filed September 24, 1993 which is hereby incorporated by reference into this application.

Within this application several publications are referenced by arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Infectious laryngotracheitis virus is a herpesvirus that causes a respiratory illness of varying virulence in chickens. Live attenuated ILTV vaccines are available to protect against the disease, but several reports have implicated vaccine viruses in the possible recurrence and spread of the disease (65 and 72), limiting vaccination to use in uninfected birds early in an outbreak. In order to design a more efficacious, attenuated vaccine, the genomic organization of the ILTV virus has been studied.

The ability to isolate viral DNA and clone this isolated DNA into bacterial plasmids has greatly expanded the approaches available to make viral vaccines. The methods used to make the present invention involve modifying cloned viral DNA sequences by insertions, deletions and single or multiple base changes. The modified DNA is then reinserted into the viral genome to render the virus non-pathogenic. The resulting live virus may then be used in a vaccine to elicit an immune response in a host animal and to protect the animal against a disease.

One group of animal viruses, the herpesviruses or *Herpetoviridae*. is an example of a class of viruses amenable to this approach. These viruses contain 100.000 to 200,000 base pairs of DNA as their genetic material. Importantly, several regions of the genome have been identified that are nonessential for the replication of virus *in vitro* in cell culture. Modifications in these regions of the DNA may lower the pathogenicity of the virus, i.e., attenuate the virus. For example, inactivation of the thymidine kinase gene renders human herpes simplex virus non-pathogenic (1), and pseudorabies virus of swine non-pathogenic (2).

Removal of part of the repeat region renders human herpes simplex virus non-pathogenic (3, 4). A repeat region has been identified in Marek's disease virus that is associated with viral oncogenicity (5). A region in herpesvirus saimiri has similarly been correlated with oncogenicity (6). Removal of part of the repeat region renders pseudorabies virus non-pathogenic (U.S. Patent No. 4,877,737, issued October 31, 1989). A region in pseudorabies virus has been shown to be deleted in naturally-occurring vaccine strains (7, 8) and it has been shown that these deletions are at least partly responsible for the lack of pathogenicity of these strains.

It is generally agreed that herpesviruses contain non-essential regions of DNA in various parts of the genome. Some of these regions are associated with virulence of the virus, and modification of them leads to a less-pathogenic virus, from which a vaccine may be derived.

Infectious laryngotracheitis virus (ILTV), an alpha herpesvirus (9), is an important pathogen of poultry in the USA, Europe, and Australia, responsible for egg production losses and death (10). It causes an acute disease of chickens which is characterized by respiratory depression, gasping and expectoration of bloody exudate. Viral replication is limited to cells of the respiratory tract wherein infection of the trachea gives rise to tissue erosion and hemorrhage.

In chickens, no drug has been effective in reducing the degree of lesion formation or in decreasing clinical signs. Vaccination of birds with various modified forms of the ILT virus derived by cell passage and/or tedious regimes of administration have been used to confer acceptable protection in susceptible chickens. Due to the limited degree of attenuation of current ILTV vaccines care must be taken to assure that the correct level of virus is maintained: enough to provide protection, but not enough to cause disease in the flock (11-21). Furthermore, these viruses may revert back to virulence, causing disease rather than providing protection against it.

ILTV has been analyzed at the molecular level. Restriction maps of the ILTV genome have been reported (22–26). The DNA sequence of several genes have been identified, i.e., thymidine kinase (27, 28), glycoprotein gB (27, 29, 30), ribonucleotide reductase (27, 31), capsid p40 (31, 32).

Furthermore, Shepard, et al. (53) disclosed that several genes located in the unique long region of the infectious laryngotracheitis virus genomic DNA are non-essential for viral replication.

Applicants have unexpectedly found that the unique short region of the ILT virus genomic DNA contains genes that are associated with ILTV virulence and that a deletion in those genes leads to an attenuated ILTV. Particularly, it was found that a deletion in the glycoprotein G (gG) gene of the ILT virus results in an attenuated virus, which is useful as a vaccine against subsequent attack by a virulent ILTV strains.

Applicants also found that a deletion in the glycoprotein I (gI) gene of the unique short region also attenuates the ILTV. Furthermore, it is contemplated that a deletion in the US2 gene, the UL-47 like gene, and the glycoprotein g60 gene of the unique short region will also attenuate the ILTV.

ILTV can become latent in healthy animals which makes them potential carriers

of the virus. For this reason, it is clearly advantageous to be able to distinguish animals vaccinated with non-virulent virus from animals infected with disease-causing wild-type or naturally-occurring virus. The development of differential vaccines and companion diagnostic tests has proven valuable in the management of pseudorabies disease (55). A similar differential marker vaccine would be of great value in the management of ILTV caused disease. The construction of differential diagnostics has focused on the deletion of glycoproteins. Theoretically, the glycoprotein chosen to be the diagnostic marker should have the following characteristics: (1) the glycoprotein and its gene should be non-essential for the production of infectious virus in tissue culture; (2) the glycoprotein should elicit a major serological response in the animal; and (3) the glycoprotein should not be one that makes a significant contribution to the protective immunity.

The ILT virus has been shown to specify at least four major glycoproteins as identified by monoclonal antibodies (M_r= 205K, 115K, 90K and 60K). Three glycoproteins seem to be antigenically related (M_r= 205K, 115K, and 90K) (34–36).

Three major ILT virus glycoproteins, gB (29, 30), gC (27, 51), and g60 (34, 53) have been described in the literature. These three genes have been sequenced and two of the ILTV genes have been shown to be homologous to the HSV glycoproteins gB, and gC.

Of these, it is known that the ILTV gB gene is an essential gene and would not be appropriate as deletion marker genes. Furthermore, the gC gene of herpesviruses has been shown to make a significant contribution to protective immunity as a target of neutralizing antibody (56) and as a target of cell-mediated immunity (57). Therefore, the gC gene is not desirable as a deletion marker gene.

As to other glycoprotein encoding genes cited above, it is not known whether

or not they would be suitable candidates for deletion in order to construct a recombinant ILT virus which can be used as a diagnostic vaccine.

Applicants have unexpectedly found that there are two glycoprotein encoding genes located within the unique short region of the ILT viral genome which could be safely deleted in order to construct a recombinant ILT virus that can be used as a diagnostic vaccine. These are the glycoprotein gG gene and the glycoprotein gI gene. By genetically engineering an ILT virus with a deletion in the glycoprotein G gene or the glycoprotein I gene, a ILT virus is produced which does not express any glycoprotein G or glycoprotein I. None of the prior arts teach or suggest that these two genes in the unique short region of the virus are appropriate candidates for deletion in order to create a diagnostic ILT virus vaccine. Although several of the herpesviruses have been genetically engineered, no examples of recombinant ILTV have been reported.

The ability to engineer DNA viruses with large genomes, such as vaccinia virus and the herpesviruses, has led to the finding that these recombinant viruses can be used as vectors to deliver vaccine antigens and therapeutic agents for animals. The herpesviruses are attractive candidates for development as vectors because their host range is primarily limited to a single target species (37) and they have the capacity for establishing latent infection (38) that could provide for stable *in vivo* expression of a foreign gene. Although several herpesvirus species have been engineered to express foreign gene products, recombinant infectious laryngotracheitis viruses expressing foreign gene products have not been constructed. The infectious laryngotracheitis viruses described above may be used as vectors for the delivery of vaccine antigens from microorganisms causing important poultry diseases. Other viral antigens which may be included in a multivalent vaccine with an ILTV vector include infectious bronchitis virus (IBV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), and Marek's disease virus (MDV). Such multivalent recombinant viruses would protect against ILT disease as well as other diseases. Similarly the infectious laryngotracheitis viruses may be used as vectors for the delivery of therapeutic

agents. The therapeutic agent that is delivered by a viral vector of the present invention must be a biological molecule that is a by-product of ILTV replication. This limits the therapeutic agent in the first analysis to either DNA. RNA or protein. There are examples of therapeutic agents from each of these classes of compounds in the form of anti-sense DNA, anti-sense RNA (39). ribozymes (40), suppressor tRNAs (41), interferon-inducing double stranded RNA and numerous examples of protein therapeutics, from hormones, e.g.. insulin, to lymphokines, e.g., interferons and interleukins, to natural opiates. The discovery of these therapeutic agents and the elucidation of their structure and function does not necessarily allow one to use them in a viral vector delivery system, however, because of the experimentation necessary to determine whether an appropriate insertion site exists.

ILTV is classified as an alpha herpesvirus with a type D genome (78) composed of a unique long region and a unique short region flanked by inverted repeats. A genomic restriction map of an Australian ILTV isolate (SA-2) was described by Johnson *et al.* (66). Using this map, Guo *et al.* (62) isolated and sequenced a DNA fragment from the USDA challenge strain which appeared to be derived from the unique short region. Applicants map the USDA challenge strain of ILTV, and reports characteristics of the putative genes present in the unique short region. The map disclosed herewith indicates that the sequence identified by Guo *et al.* (62) is part of the short repeat sequence, and is not from the unique short. Other reports (69 and 70) describe the sequences of two genes, one homologous to PRV gG and the other unlike other reported herpesvirus genes. These two genes were mapped to the unique long region of SA-2. However, these sequences are identical to sequences identified in this application as being from the unique short region. The data in this application indicate that the overall organization of the short region of ILTV is similar to other herpesviruses.

SUMMARY OF THE INVENTION

The present invention provides a recombinant, attenuated infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene. This attenuated virus is useful as a vaccine against infectious laryngotracheitis virus.

The present invention also provides a recombinant, attenuated infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the US2 gene, UL47-like gene, ORF4 gene or glycoprotein g60 gene.

The present invention also provides a method for distinguishing chickens or other poultry vaccinated with a recombinant infectious laryngotracheitis virus which produces no glycoprotein gG from those infected with a naturally-occuring infectious laryngotracheitis virus.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1H:

The nucleotide sequence of 13,473 base pairs of contiguous DNA from the unique short region of the ILT virus. This sequence contains the entire 13,098 base pair unique short region as well as 273 base pairs of repeat region at one end and 102 base pairs of repeat region at the other end. The nucleotide sequences of Figures 1A-1H begin with the internal repeat sequence and end within the terminal repeat sequence. The unique short region begins at base pair 274 of this Figure. Sequence ID NO:59 contains the nucleotide sequence of 18,912 base paris of contiguous DNA from the unique short and repeat regions of the ILT virus. This sequence contains the entire 13,094 base pair unique short region as well as 2909 base paris of internal repeat region and 2909 base paris of short terminal repeat region. The nucleotide sequences begin with the internal repeat sequence and end within the terminal repeat sequence. The unique short region begins at base pair 2910.

Figure 2:

*Asp*718 I restriction enzyme map of the infectious laryngotracheitis virus (ILTV) USDA 83-2 genome. The upper diagram identifies the unique long (U_L), internal repeat (IR), unique short (U_S), and terminal repeat (TR) sections found in the ILTV genome. A map of the *Asp*718 I restriction endonuclease sites in the ILTV genome is shown below. Letters A through O identify *Asp*718 I restriction endonuclease fragments with "A" representing the largest fragment. Fragment "L" is the 2.5 kb *Asp*718 I fragment, fragment "H" is the 5164 bp *Asp*718 I fragment, and fragment

"G" is the 8.0 kb *Asp*718 I fragment. The fragments marked with asterisks contain a hypervariable region of approximately 900 bp that is repeated from one to 12 times. Since no one size predominates, these fragments appear in submolar amounts that are not well resolved on an ethidium bromide stained gel. The position of these repeats is indicated in the Figures by the crooked dashed lines.

Figure 3: Open reading frames within the unique short region of infectious laryngotracheitis virus (ILTV) USDA 83-2. The 13,473 base pairs of the short region of ILTV contains the entire 13,098 base pair unique short region as well as 273 base pairs of repeat region at one end and 102 base pairs of repeat region at the other end. The unique short region contains 13 methionine initiated open reading frames (ORF) of greater than or equal to 110 amino acids (excluding smaller nested ORFs). All 13 ORFs were aligned to the Entrez release 6.0 virus division of the Genbank DNA database utilizing the IBI MacVector Protein to DNA alignment option (default settings). Eight of the ORFs exhibited significant homology to one or more other virus genes: unique short (US2), protein kinase (PK), unique long 47-like (UL47-like), and glycoproteins gG, g60, gD, gI, and gE.

Figures 4A-4B:

Detailed description of the DNA insertion in Homology Vector 472-73.27. Diagram showing the orientation of DNA fragments assembled in plasmid 472-73.27. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 20, 21, 22 and 23). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The

location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, infectious laryngotracheitis virus (ILTV) , human cytomegalovirus immediate early (HCMV IE), pseudorabies virus (PRV), lactose operon Z gene (lacZ), *Escherichia coli* (E. coli), polyadenylation signal (poly A), and base pairs (BP).

Figures 5A-5B:

Detailed description of the DNA insertion in Homology Vector 501-94. Diagram showing the orientation of DNA fragments assembled in plasmid 501-94. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 24, 25, 26, and 27). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction.

The following abbreviations are used, infectious laryngotracheitis virus (ILTV) , human cytomegalovirus immediate early (HCMV IE), pseudorabies virus (PRV), lactose operon Z gene (lacZ), *Escherichia coli* (E. coli), polyadenylation signal (poly A), thymidine kinase (TK), and base pairs (BP).

Figures 6A-6B:

Detailed description of the DNA insertion in Homology Vector 544-55.12. Diagram showing the orientation of DNA fragments assembled in plasmid 544-55.12. The origin of each fragment

is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 28. 29, 30, and 31). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, infectious laryngotracheitis virus (ILTV), herpes simplex virus type 1 (HSV-1), pseudorabies virus (PRV), β–glucuronidase gene (uidA), *Escherichia coli* (E. coli), polyadenylation signal (poly A), and base pairs (BP).

Figures 7A-7C:

Detailed description of the DNA insertion in Homology Vector 562-61.1F. Diagram showing the orientation of DNA fragments assembled in plasmid 562-61.1F. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 32, 33, 34 35, 36 and 37). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, infectious laryngotracheitis virus (ILTV), herpes simplex virus type 1 (HSV-1), pseudorabies virus (PRV), β–glucuronidase gene (uidA), *Escherichia coli* (E. coli), polyadenylation signal (poly A), and base pairs (BP).

Figures 8A-8C:

Detailed description of the DNA insertion in Homology Vector

560-52.F1. Diagram showing the orientation of DNA fragments assembled in plasmid 560-52.F1. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 38, 39, 40, 41, and 42). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, infectious laryngotracheitis virus (ILTV) , herpes simplex virus type 1 (HSV-1), pseudorabies virus (PRV), β–glucuronidase gene (uidA), *Escherichia coli* (E. coli), polyadenylation signal (poly A), unique long 47 (UL47-like), open reading frame 4 (ORF4), glycoprotein G (gG), and base pairs (BP).

Figures 9A-9B:

Detailed description of the DNA insertion in Homology Vector 579-14.G2. Diagram showing the orientation of DNA fragments assembled in plasmid 579-14.G2. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 43, 44, 45, and 46). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, infectious laryngotracheitis virus (ILTV), herpes simplex virus type 1 (HSV-1), pseudorabies virus (PRV), β–glucuronidase gene (uidA), *Escherichia coli* (E.

coli), polyadenylation signal (poly A), and base pairs (BP).

Figures 10A-10B:

Detailed description of the DNA insertion in Plasmid Vector 544-39.13. Diagram showing the orientation of DNA fragments assembled in plasmid 544-39.13. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 47, 48, 49, and 50). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the remnants of sites which were destroyed during construction. The following abbreviations are used, pseudorabies virus (PRV), β–glucuronidase gene (uidA), *Escherichia coli* (E. coli), herpes simplex virus type 1 (HSV-1), polyadenylation signal (poly A), and base pairs (BP).

Figures 11A-11C:

Detailed description of the DNA insertion in Plasmid Vector 388-65.2. Diagram showing the orientation of DNA fragments assembled in plasmid 388-65.2. The origin of each fragment is indicated in the table. The sequences located at each of the junctions between fragments are also shown (SEQ ID NO's: 51, 52, 53, and 54). The restriction sites used to generate each fragment as well as the synthetic linker sequences which were used to join the fragments are described for each junction. The synthetic linker sequences are underlined by a heavy bar. The location of several gene coding regions and regulatory elements is also given. Restriction sites in brackets [] indicate the

remnants of sites which were destroyed during construction. The following abbreviations are used, human cytomegalovirus immediate early (HCMV IE), lactose operon Z gene (lacZ), *Escherichia coli* (E. coli), pseudorabies virus (PRV), polyadenylation signal (poly A), and base pairs (BP).

Figure 12: The genome of the ILTV virus, identifying the unique long (UL), unique short (US), internal repeat (IR), and terminal repeat (TR) is shown. The *Bam*HI, *Asp*718I, *Not*I, and *Sfi*I restriction maps of the virus are drawn underneath, with the highly repetitive region of the short repeats indicated by a set of wavy lines. The position of the cosmids used to determine the map of ILTV are drawn beneath the restriction map. Note that cosmid 2F12 contains two non-contiguous sections. Three probes used to characterize the ILTV genome are indicated as P1, P2, and P3. P1 is a 0.9 kb *Not*I fragment found at the terminus of the unique long region, P2 is the 856 bp *Hin*dIII fragment found in multiple copies within the short repeat, and P3 Is a 6.6 kb *Not*I fragment used to identify the fragments at the end of the terminal repeat.

Figure 13: The region sequenced, and the positions of the *Asp*718I, *Bam*HI, *Not*I, and *Sfi*I sites are shown. The and extent and orientation of the open reading frames found in the ILTV unique short and the flanking short repeat regions are indicated.

Figure 14: Southern blot showing the repetition of an 856 bp element within the short repeat. Genomic ILTV DNA digested with *Sfi*I (a), *Hin*dIII (b), *Not*I (c), *Asp*718I (d), or *Bam*HI (e) was probed with an 856 bp *Hin*dIII fragment from the short repeat. Positions of molecular weight markers are indicated.

Figure 15: Depiction of the position of the 856 bp repeat region in the USDA strain, compared to the same region from the SA-2 strain as described by Johnson *et al.* Three repeats are arbitrarily shown in the USDA strain, the region is not repeated in SA2. B=*Bam*HI, H=*Hin*dIII, R=856 bp repeat.

Figure 16: Southern blot identifying fragments from the internal and terminal repeat that hybridized to a 6.6 kb *Not*I fragment containing the junction of the unique long and the internal repeat. Genomic ILTV DNA digested with *Not*I (a), *Asp*718I (b), and *Bam*HI (c) was probed with the 6.6 kb *Not*I fragment. Positions of molecular weight markers are indicated.

Figure 17: The relationship of herpesvirus UL47 proteins to each other and to the ILTV UL47 homolog in a conserved region. Amino acids shared between ILTV UL47 and the other UL47 proteins are in boldface type. Pairwise comparisons have been made between the sequences as shown. A vertical bar indicates an identical amino acid, two dots indicate a positive probable acceptable mutation rate and one dot indicates a neutral probable acceptable mutation rate (60).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region of the viral genome, wherein the deletion is in the glycoprotein gG gene. Said deletion attenuates the virus, rendering it suitable for use as a vaccine against infectious laryngotracheitis virus. A preferred embodiment of this invention is a recombinant infectious laryngotracheitis designated S-ILT-014 (ATCC Accession No. 2427). The S-ILT-014 virus has been deposited pursuant to the Budapest Treaty on the International Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. on September 22, 1993 under ATCC Accession No. 2427). Another preferred embodiment of this invention is a recombinant infectious laryngotracheitis virus designated S-ILT-002.

For purposes of this invention, "a recombinant infectious laryngotracheitis virus" is a live infectious laryngotracheitis virus which has been generated by the recombinant methods well known to those of skill in the art, e.g., the methods set forth in DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS in Materials and Methods, and the virus has not had genetic material essential for the replication of the infectious laryngotracheitis virus deleted.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene and a deletion in the US2 gene. One preferred embodiment of this invention is a recombinant infectious laryngotracheitis virus designated S-ILT-009.

The present invention further provides a recombinant laryngotracheitis virus

comprising the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene and a deletion in the ORF4 gene.

The present invention further provides a recombinant infectious laryngotracheitis virus which comprises the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene and a deletion in the UL47-like gene.

The present invention further provides a recombinant infectious laryngotracheitis virus which comprises the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene, a deletion in the ORF4 gene, and a deletion in the UL47-like gene. A preferred embodiment of this invention is a recombinant infectious laryngotracheitis virus designated S-ILT-015.

The present invention further provides a recombinant infectious laryngotracheitis virus which comprises the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene and a deletion in the glycoprotein g60 gene. A preferred embodiment of this invention is a recombinant infectious laryngotracheitis virus designated S-ILT-017.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the glycoprotein gG gene and a deletion in the glycoprotein gI gene.

The present invention further provides a recombinant infectious laryngotracheitis virus which comprises the infectious laryngotracheitis viral genome containing a deletion in the glycoprotein gG gene and a deletion in the thymidine kinase (TK) gene.

The present invention further provides a recombinant infectious

laryngotracheitis virus comprising the infectious laryngotracheitis virus genome which contains a deletion in the unique short region of the viral genome, wherein the deletion in the glycoprotein gG gene, and which also contains an insertion of a foreign gene. The foreign gene is inserted into a non-essential site of the infectious laryngotracheitis viral genome in such a way that it is capable of being expressed in a recombinant infectious laryngotracheitis infected host cell.

For purposes of this invention, "a non-essential site" of the infectious laryngotracheitis viral genome is a region of the viral genome which is not necessary for viral infection and replication.

The following non-essential sites of the infectious laryngotracheitis viral genome are preferred sites for inserting a foreign gene into the virus : the thymidine kinase (TK) gene, the US2 gene, the UL47-like gene, the ORF4 gene, the glycoprotein gG gene, the glycoprotein g60 gene, and the glycoprotein gI gene.

The foreign gene, which is inserted into a non-essential site in the infectious laryngotracheitis viral genome, may encode a screenable marker, such as *E. coli* *B*-galactosidase or *E. coli* *B*-glucuronidase.

The foreign gene which is inserted into a non-essential site in the infectious laryngotracheitis viral genome, may encode an antigenic polypeptide which, when introduced into the host cell, induces production of protective antibodies against an avian disease causing agent from which the antigen is derived or derivable. Antigenic polypeptide which includes, but is not limited to: marek's disease virus (MDV) gA, marek's disease virus gB, marek's disease virus gD, Newcastle disease virus (NDV) HN, Newcastle disease virus F, infectious laryngotracheitis virus (ILT) gB, infectious laryngotracheitis virus gI, infectious laryngotracheitis virus gD, infectious bursal disease virus (IBDV) VP2, infectious bursal disease virus VP3, infectious bursal disease virus VP4,

infectious bursal disease virus polyprotein, infectious bronchitis virus (IBV) spike, infectious bronchitis virus matrix, avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia virus, *Salmonella spp. E. coli*, *Pasteurella spp.*, *Bordetella spp.*, *Eimeria spp.*, *Histomonas spp.*, *Trichomonas spp.*, Poultry nematodes, cestodes, trematodes, poultry mites/lice, and poultry protozoa.

In one embodiment of the recombinant infectious laryngotracheitis virus the foreign DNA sequence encodes a cytokine. In another embodiment the cytokine is chicken myelomonocytic growth factor (cMGF) or chicken interferon (cIFN). Cytokines include, but are not limited to: transforming growth factor beta, epidermal growth factor family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte protease inhibitor, stem cell factor, tumor necrosis factors, and soluble TNF receptors. These cytokines are from humans, bovine, equine, feline, canine, porcine or avian. Recombinant ILT virus expressing cytokines is useful to enhance the immune response when combined with vaccines containing anitgens of disease causing microorganisms.

Recombinant infectious laryngotracheitis virus expressing cytokines is used to enhance the immune response either alone or when combined with vaccines containing cytokines or antigen genes of disease causing microorganisms.

Antigenic polypeptide of a human pathogen which are derived from human

herpesvirus include, but are not limited to: hepatitis B virus and hepatitis C virus hepatitis B virus surface and core antigens, hepatitis C virus, human immunodeficiency virus, herpes simplex virus-1, herpes simplex virus-2, human cytomegalovirus, Epstein-Barr virus, Varicella-Zoster virus, human herpesvirus-6, human herpesvirus-7, human influenza, measles virus, hantaan virus, pneumonia virus, rhinovirus, poliovirus, human respiratory syncytial virus, retrovirus, human T-cell leukemia virus, rabies virus, mumps virus, malaria (*Plasmodium falciparum*), *Bordetella pertussis*, Diptheria, *Rickettsia prowazekii*, *Borrelia berfdorferi*, Tetanus toxoid, malignant tumor antigens.

The antigenic polypeptide of an equine pathogen is derived from equine influenza virus, or equine herpesvirus. In one embodiment the antigenic polypeptide is equine influenza neuraminidase or hemagglutinin. Examples of such antigenic polypeptide are: equine influenza virus type A/Alaska 91 neuraminidase and hemagglutinin, equine influenza virus type A/Prague 56 neuraminidase and hemagglutinin, equine influenza virus type A/Miami 63 neuraminidase, equine influenza virus type A/Kentucky 81 neuraminidase and hemagglutinin, equine herpesvirus type 1 glycoprotein B, and equine herpesvirus type 1 glycoprotein D, *Streptococcus equi*, equine infectious anemia virus, equine encephalitis virus, equine rhinovirus and equine rotavirus.

The antigenic polypeptide of an equine pathogen is derived from bovine respiratory syncytial virus or bovine parainfluenza virus, and is capable of being expressed in a host infected by the recombinant infectious bovine rhinotracheitis virus. For example, the antigenic polypeptide is derived from bovine respiratory syncytial virus attachment protein (BRSV G), bovine respiratory syncytial virus fusion protein (BRSV F), bovine respiratory syncytial virus nucleocapsid protein (BRSV N), bovine parainfluenza virus type 3 fusion protein, and the bovine parainfluenza virus type 3 hemagglutinin neuraminidase.

The foreign gene may be put under control of an endogenous upstream infectious laryngotracheitis virus promoter, or it may be put under control of

a heterologous upstream promoter. The heterologous upstream promoter may be derived from the HCMV IE promoter, the PRV gX promoter, and BHV-1.1 VP8 promoter.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion or other alteration in the unique short region of the viral genome, wherein the deletion or alteration is in the glycoprotein gG gene, so that upon replication, the recombinant virus produces no glycoprotein gG. The following recombinant viruses are preferred embodiments of this invention: A recombinant infectious laryngotracheitis virus designated S-ILT-002, S-ILT-014, S-ILT-009, S-ILT-015, and S-ILT-017.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion or other alteration in the unique short region of the viral genome, wherein the deletion or alteration is in the glycoprotein gI gene, so that upon replication, the recombinant virus produces no glycoprotein gI.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion or other alteration in the unique short region of the viral genome, wherein the deletion or alteration is in the glycoprotein gG gene and in the glycoprotein gI gene, so that upon replication, the recombinant virus produces no glycoprotein gG and no glycoprotein gI.

The present invention further provides a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region of the viral genome, wherein the deletion is in the US2 gene, UL47-like gene, glycoprotein g60 gene. It is contemplated that a deletion in any one of these genes will attenuate the virus, rendering it suitable to be used as a vaccine against infectious

laryngotracheitis virus.

The present invention further provides a recombinant infectious laryngotracheitis virus which comprises a foreign gene inserted within the unique short region of the infectious laryngotracheitis viral genome, provided, however, that the insertion is not in the protein kinase gene, the glycoprotein gD gene, the glycoprotein gE gene and the ORF10 gene. The foreign gene is inserted in such a way that it is capable of being expressed in the recombinant infectious laryngotracheitis virus infected host cell. Preferred insertion sites are the US2 gene, the UL47-like gene, the ORF4 gene and the glycoprotein g60 gene.

A foreign gene may be inserted within any one of these sites in such a way that it may be expressed in a host cell which is infected which the recombinant infectious laryngotracheitis virus of the present invention.

The foreign gene thus inserted may encode a screenable marker, such as E. coli ß-galactosidase or *E. coli* ß-glucuronidase.

The foreign gene thus inserted may encode an antigenic polypeptide which, when introduced into the host cell, induces production of protective antibodies against an avian disease causing agent from which the antigen is derived or derivable. Such antigenic polypeptide may be derived or derivable from infectious bronchitis virus, Newcastle disease virus, infectious bursal disease virus, and Marek's disease virus. Such antigenic polypeptide may also be derived or derivable from avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia agent, *Salmonella spp. E. coli, Pasterurella spp., Bordetella spp. Eimeria spp. Histomonas spp., Trichomonas spp*, Poultry nematodes, cestodes, trematodes, poultry mites/lice, poultry protozoa.

The foreign gene thus inserted may be put under control of an endogenous upstream infectious laryngotracheitis virus promoter, or it may be put under control of a heterologous upstream promoter. The heterologous upstream promoter may be the HCMV IE promoter, the PRV gX promoter or BHV-1.1 VP8 promoter.

The present invention further provides a vaccine for infectious laryngotracheitis virus which comprises a suitable carrier and an effective immunizing amount of any of the recombinant infectious laryngotracheitis virus of the present invention. This vaccine may contain either inactivated or live recombinant virus.

Suitable carriers for the recombinant virus are well known in the art and include proteins, sugars, etc. One example of such a suitable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as hydrolyzed proteins, lactose, etc. Preferably, the live vaccine is created by taking tissue culture fluids and adding stabilizing agents such as stabilizing, hydrolyzed proteins. Preferably, the inactivated vaccine uses tissue culture fluids directly after inactivation of the virus.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region of the viral genome, wherein the deletion is in the glycoprotein gG gene. A preferred embodiment of this invention is a vaccine which comprises a suitable carrier and an effective immunizing amount of any one of the following viruses: recombinant infectious laryngotracheitis viruses designated S-ILT-014, S-ILT-002, S-ILT-009, S-ILT-015 and S-ILT-017.

The present invention further provides a multivalent vaccine for infectious laryngotracheitis virus and for one or more of other avian diseases which

comprises an effective immunizing amount of a recombinant virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region, wherein the deletion is in the glycoprotein gG gene, and an insertion of a foreign gene into a non-essential site of the viral genome.

The foreign gene encodes an antigenic polypeptide which induces host cell production of protective antibodies against an avian disease causing agent from which the antigen is derived or derivable.

The foreign gene may be derived or derivable from infectious bronchitis virus, Newcastle disease virus, infectious bursal disease virus, and Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia agent, *Salmonella spp.*, *E. coli*, *Pasteurella spp.*, *Bordetella spp.*, *Eimeria spp.*, *Histomonas spp.*, *Trichomonas spp.*, poultry nematodes, cestodes, trematodes, poultry mites/lice, poultry protozoa.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome containing a deletion or other alteration in the unique short region of the viral genome, wherein the deletion or alteration is in the glycoprotein gG gene, so that upon replication, the recombinant virus produces no glycoprotein gG. A preferred embodiment of this invention is a vaccine which comprises a suitable carrier and an effective immunizing amount of any one of the following viruses: recombinant infectious laryngotracheitis viruses designated S-ILT-014, S-ILT-002, S-ILT-009, S-ILT-015 and S-ILT-017.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion or other alteration in the unique short region of the

viral genome, wherein the deletion or alteration is in the glycoprotein gI gene so that upon replication, the recombinant virus produces no glycoprotein gI.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion or other alteration in the unique short region of the viral genome, wherein the deletion or alteration is in the glycoprotein gG gene and the glycoprotein gI gene so that upon replication, the recombinant virus produces no glycoprotein gG and glycoprotein gI.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region of the viral genome, wherein the deletion is in the US2 gene, UL47-like gene, or glycoprotein g60 gene.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains a deletion in the unique short region of the viral genome, wherein the deletion is in the US2 gene, ORF4 gene, UL47-like gene, or glycoprotein g60 gene, and insertion of a foreign gene into a non-essential site in the viral genome.

The foreign gene encodes an antigenic polypeptide which induces host cell production of protective antibodies against an avian disease causing agent from which the antigen is derived or derivable.

The foreign gene may be derived or derivable from infectious bronchitis virus, Newcastle disease virus, infectious bursal disease virus, and Marek's disease

virus, avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia agent, *Salmonella spp.*, *E. coli*, *Pasteurella spp.*, *Bordetella spp.*, *Eimeria spp.*, *Histomonas spp.*, *Trichomonas spp.*, poultry nematodes, cestodes, trematodes, poultry mites/lice, poultry protozoa.

The present invention further provides a vaccine which comprises a suitable carrier and an effective immunizing amount of a recombinant infectious laryngotracheitis virus comprising the infectious laryngotracheitis viral genome which contains an insertion of a foreign gene into a non-essential site in the viral genome. The foreign gene encodes an antigenic polypeptide which induces host cell production of protective antibodies against an avian disease causing agent from which the antigen is derived or derivable.

The foreign gene may be derived or derivable from infectious bronchitis virus, Newcastle disease virus, infectious bursal disease virus, and Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia agent, *Salmonella spp. E. coli, Pasterurella spp.*, *Bordetella spp. Eimeria spp. Histomonas spp.*, *Trichomonas spp*, Poultry nematodes, cestodes, trematodes, poultry mites/lice, poultry protozoa.

The present invention further provides a method of immunizing an animal against infectious laryngotracheitis virus which comprises administering to chickens or other poultry an effective immunizing dose of any of the vaccines of the present invention.

The present invention further provides a method for distinguishing chickens or other poultry which are vaccinated with an effective immunizing amount of a recombinant virus which produces no glycoprotein gG from those which are infected with a naturally-occurring infectious laryngotracheitis virus. This method comprises analyzing a sample of body fluid from the chickens or other

poultry for the presence of glycoprotein gG of the infectious laryngotracheitis virus and at least one other antigen normally expressed in chickens or other poultry infected by a naturally-occurring infectious laryngotracheitis virus. The presence of antigen which is normally expressed in chickens or other poultry infected by a naturally-occurring infectious laryngotracheitis virus and the absence of glycoprotein gG in the body fluid is indicative of being vaccinated with the recombinant vaccine and not infected with a naturally-occurring infectious laryngotracheitis virus. The presence of glycoprotein gG and the antigen in the body fluid may be determined by detecting in the body fluid antibodies specific for the antigen and glycoprotein gG.

The present invention further provides a method for distinguishing chickens or other poultry which are vaccinated with an effective immunizing amount of a recombinant infectious laryngotracheitis virus which produces no glycoprotein gI from those which are infected with a naturally-occurring infectious laryngotracheitis virus. This method comprises analyzing a sample of body fluid from the chickens or other poultry for the presence of glycoprotein gI of the infectious laryngotracheitis virus and at least one other antigen normally expressed in chickens or other poultry infected by a naturally-occurring infectious laryngotracheitis virus. The presence of the antigen which is normally expressed in chickens or other poultry infected by a naturally-occurring infectious laryngotracheitis virus and the absence of glycoprotein gI in the body fluid is indicative of being vaccinated with the recombinant vaccine and not infected with a naturally-occurring infectious laryngotracheitis virus. The presence of the antigen and glycoprotein gI in the body fluid may be determined by detecting in the body fluid antibodies specific for the antigen and glycoprotein gI.

The present invention further provides a method for distinguishing chickens or other poultry which are vaccinated with an effective immunizing amount of a recombinant virus which produces no glycoprotein gG and no glycoprotein gI from those which are infected with a naturally-occurring infectious

laryngotracheitis virus. This method comprises analyzing a sample of body fluid from the chickens or other poultry for the presence of glycoprotein gG and gI of the infectious laryngotracheitis virus and at least one other antigen normally expressed in an animal infected by a naturally-occurring infectious laryngotracheitis virus. The presence of the antigen which is normally expressed in chickens or other poultry by a naturally-occurring infectious laryngotracheitis virus and the absence of glycoprotein gG and gI in the body fluid is indicative of being vaccinated with the vaccine and not infected with a naturally-occurring infectious laryngotracheitis virus. The presence of the antigen and glycoprotein gG and gI in the body fluid may be determined by detecting in the body fluid antibodies specific for the antigen and glycoprotein gG and gI.

The present invention further provides a homology vector for producing a recombinant infectious laryngotracheitis virus by inserting a foreign DNA into the unique short region of the infectious laryngotracheitis genomic DNA, which comprises a double-stranded DNA molecule consisting essentially of a double-stranded foreign gene, which is flanked on either side by the double-stranded DNA homologous to the DNA located in the unique short region of the genomic DNA, provided, however, that the flanking sequences are not homologous to the glycoprotein gD gene, the glycoprotein gE gene, the protein kinase gene, and the ORF10 gene. The foreign gene may encode a screenable marker, such as *E. coli B*-galactosidase or *E. coli B*-glucuronidase.

The present invention further provides a homology vector for producing a recombinant infectious laryngotracheitis virus by deleting DNA which encodes a screenable marker, which has been inserted into the infectious laryngotracheitis virus genomic DNA, which comprises a double stranded DNA molecule consisting essentially of a double-stranded DNA to be deleted, which is flanked on each side by a double stranded DNA homologous to the infectious laryngotracheitis virus glycoprotein gG gene, glycoprotein gI gene, US2 gene, or UL-47 like gene. Preferred embodiments of this invention are the homology

vectors designated Homology Vector 544-55.12, Homology Vector 562-61.1F, Homology Vector 472-73.27, Homology Vector 560-52.F1 and Homology Vector 579-14.G2.

This invention provides an isolated nucleic acid molecule encoding a US10 gene (SEQ ID NOs:60 and 70), AvSp gene (SEQ ID NOs: 61 and 71), US2 gene (SEQ ID NO:62), PK gene (SEQ ID NO:63), UL47 gene (SEQ ID NO:64), gG gene (SEQ ID NO:65), ORF5 gene (SEQ ID NO: 66), gD gene (SEQ ID NO:67), gI gene (SEQ ID NO:68), gE gene (SEQ ID NO:69), or ORF9 gene (SEQ ID NO:70).

This invention provides an isolated polypeptide encoded by the US10 gene (SEQ ID NOs:60 and 70), AvSp gene (SEQ ID NOs: 61 and 71), US2 gene (SEQ ID NO:62), PK gene (SEQ ID NO:63), UL47 gene (SEQ ID NO:64), gG gene (SEQ ID NO:65), ORF5 gene (SEQ ID NO: 66), gD gene (SEQ ID NO:67), gI gene (SEQ ID NO:68), gE gene (SEQ ID NO:69), or ORF9 gene (SEQ ID NO:70).

EXPERIMENTAL DETAILS

Materials and Methods

PREPARATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS STOCK SAMPLES. Infectious laryngotracheitis virus stock samples were prepared by infecting primary chicken embryo kidney cells (CEK: obtained from Spafas, Inc.) or primary chicken kidney cells (CK; obtained from chicks hatched from fertile eggs supplied by Hyvac) (50) in 225 cm^2 flasks with 0.5 ml of viral stock containing 10^5-10^6 pfu in 1X Eagle's Basal Medium (modified) with Hank's salts (BME), 10% bromoethylamine(BEI)-treated fetal bovine serum (FBS), 1% glutamine stock, 2% pennicillin/streptomycin (P/S) stock, and 1% sodium bicarbonate stock (these components are obtained from Irvine Scientific or an equivalent supplier, and hereafter the growth medium is referred to as complete BME medium). Viral stocks were then harvested 4-5 days later. Infected media and cells were resuspended in complete medium containing 20% sterile whole milk and stored frozen at -70°C.

PREPARATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS DNA. Four to five days after viral infection, cells and media were scraped from each flask into 15 ml conical centrifuge tubes and pelleted at 1700 x g for 5 minutes at 4°C. Because as much as 50% of the virus may be in the media, the supernatants were saved and treated as will be described below. The cell pellets were resuspended in 1 ml PBS per tube, combined and centrifuged again at 1700 x g for 5 minutes. The pellets were resuspended in 1 ml/flask of a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 1.5 mM $MgCl_2$ and were incubated for 15 minutes at 4°C. Twenty five µls of 20% NP40 per flask was added, and the mixture was then homogenized in a dounce homogenizer using an A pestle. The preparation was centrifuged at 1700 x g for 10 minutes at 4°C and the supernatant was retained. Ten µl of 0.5 M EDTA, 50 µl of 20% SDS, and 25 µl of 10 mg/ml proteinase K was added to the supernatant (per original flask). In some cases, this was then combined with virus obtained from

the cell media supernatants (see above). The mixture was then treated at 65°C for 1-16 hours, followed by two extractions with phenol saturated with 100 mM Tris-HCl, pH 8. DNA in the aqueous phase was then precipitated with added 3 M sodium acetate (1/10th volume) and 2.5 vols of 100% ethanol.

To obtain virus from the media, the cell media supernatants were centrifuged at 23,500 x g for 30 minutes, and drained well. The pellet was resuspended in the above proteinase K-containing mixture as described. The DNA pellets were resuspended in 20 µl TE/flask and could be used at this point for further experiments or treated further to remove RNA with pancreatic RNase A, followed by phenol extraction and ethanol precipitation to obtain the DNA.

To prepare viral DNA minipreps, infected 10 cm. dishes were scraped into conical centrifuge tubes and centrifuged 5 minutes at 1000 x g. Cell media supernatants were kept and treated as above. The cell pellets were each resuspended in 0.5 ml of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% NP40, and incubated 10 minutes at room temperature. Ten µl of 10 mg/ml RNase A was added, and the preparation was centrifuged 5 minutes at 1000 x g. Twenty-five µl of 20 % SDS and 25 µl of 10 mg/ml proteinase K was added to the supernatant, and the entire preparation was added to the viral pellet from the cell media if it was used. The mixture was incubated at 55-65°C for one hour, extracted with buffer-saturated phenol and precipitated by the addition of 1 ml of ethanol. The DNA pellet was resuspended in 20 µl of TE and stored at 4°C.

POLYMERASE FILL-IN REACTION. DNA was resuspended in buffer containing 50 mM Tris pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 400 micromolar each of the four deoxyribonucleotides. Ten units of Klenow DNA polymerase (Gibco BRL) were added and the reaction was allowed to proceed for 15 minutes at room temperature. The DNA was phenol extracted and ethanol precipitated as above.

DNA SEQUENCING. Sequencing was performed using the Sequenase Kit (US

Biochemicals) and α^{35}S-dATP (New England Nuclear). Reactions using both the dGTP mixes and the dITP mixes were performed to clarify areas of compression. Alternatively, compressed areas were resolved on formamide gels. Templates were double-stranded plasmid subclones or single stranded M13 subclones, and primers were either made to the vector just outside the insert to be sequenced, or to previously obtained sequence. Sequence obtained was assembled and compared using Dnastar software. Manipulation and comparison of sequences obtained was performed with IBI MacVector, Superclone and Supersee Align programs from Coral Software.

MOLECULAR BIOLOGICAL TECHNIQUES. Techniques for the manipulation of bacteria and DNA, including such procedures as digestion with restriction endonucleases, gel electrophoresis, extraction of DNA from gels, ligation, phosphorylation with kinase, treatment with phosphatase, growth of bacterial cultures, transformation of bacteria with DNA, and other molecular biological methods are described (42, 43). The polymerase chain reaction (PCR) was used to introduce restriction sites convenient for the manipulation of various DNAs (44). In general amplified fragments were less than 500 base pairs in size and critical regions of amplified fragments were confirmed by DNA sequencing. Except as noted, these techniques were used with minor variation.

SOUTHERN BLOTTING OF DNA. The general procedure for Southern blotting was taken from Maniatis *et al.* (1982) and Sambrook, et.al.(1989) (42, 43). DNA was blotted to nylon membrane (Biorad Zetaprobe) in 0.4M NaOH and prehybridized for 5 minutes in a solution containing 0.25 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% SDS at 65°C. Labeled probe was added that had been labeled by random priming using a Genius™ non-radioactive labeling kit from Boehringer-Mannheim. Hybridization was overnight at 65°C. Filters were washed twice with 40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 5% SDS and then twice with 40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS for 30 minutes each at 65°C. Detection of bound probe was performed using the Boehringer

Mannheim Genius™ non-radioactive detection kit.

DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The method is based upon the $CaCl_2$ procedure of Chen and Okayama (1987) (45) with the following modifications. Generation of recombinant ILT virus is dependent upon homologous recombination between ILT viral DNA and the plasmid homology vector containing the desired foreign DNA flanked by the appropriate herpesvirus cloned sequences. Plasmid DNA (10-40 mg) was added to 250 ml of a solution having a final concentration of 0.25 M $CaCl_2$. An equal volume of a buffer containing 50 mM MOPS (pH 6.95), 280 mM NaCl, and 1.5 mM Na_2HPO_4 was added to the DNA/$CaCl_2$ solution. After 10 minutes at room temperature, the mixture was added dropwise to a 6 cm dish of CEK cells on maintenance media, and placed at 39°C for 4 to 5 hours. The cells were rinsed once with PBS, once with 20% glycerol in PBS for 2 minutes, rinsed again with PBS and fed with maintenance media. 1.5 ml of ILT viral stock was added to the media, and the cells were incubated overnight. The next day, fresh maintenance media was added, and the cells were incubated for two more days. The transfection stock was harvested, aliquoted, and frozen at -70°C.

PROCEDURE FOR GENERATING ILTV SUBGENOMIC DNA FRAGMENTS. The ability to generate herpesviruses by cotransfection of cloned overlapping subgenomic fragments has been demonstrated for pseudorabies virus (46). If deletions and/or insertions are engineered directly into the subgenomic fragments prior to the cotransfection, this procedure results in a high frequency of viruses containing the genomic alteration, greatly reducing the amount of screening required to purify the recombinant virus. The procedure of overlapping cosmids to map restriction enzyme sites was employed.

A library of subclones containing overlapping ILTV subgenomic fragments was generated as follows. USDA ILTV Strain 83–2 has been designated S-ILT-001. Approximately 20 µg of ILTV DNA (obtained from S-ILT-001) in 0.5 ml of

10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE) was sheared by passing it twice through a 25 guage needle as previously described (46). The DNA was centrifuged through a 15-40% glycerol gradient in 50 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.3 M NaCl for 5.5 hours at 274,000 x g. Fractions were analyzed on a 0.3% agarose gel, and those containing DNA of 35-50 kb were pooled, diluted twofold with TE, and precipitated with one tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol. The tubes were centrifuged for one hour at 109,000 x g at 10°C . Pellets were resuspended, transferred to microfuge tubes, and precipitated with one tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol. The DNA was resuspended in TE. DNA ends were made blunt ended by the POLYMERASE FILL-IN REACTION. The DNA was purified by extraction with both buffer saturated phenol and ether, precipitated with sodium acetate and ethanol as above, and resuspended in TE. Half of this material was ligated with 3 mg of vector, pSY1626, by the DNA ligation reaction. The vector used was pSY1626, which was made as follows. Cosmid pHC79 (Gibco BRL) was cut with *Hind*III and *Ava*I to remove the tetracycline gene, and the ends were filled in with Klenow polymerase (FILL IN REACTION). The polylinker from pWE15 (Stratagene) was ligated into this vector. The polylinker was isolated by digestion with *Eco*RI, the ends were filled in with Klenow polymerase (FILL IN REACTION), and the fragment was purified on a LMP-agarose gel. DNA ligation was performed in the presence of melted agarose. The resulting cosmid, pSY1005, was modified at the *Eco*RI site to create pSY1626 by blunt-ended insertion of a 1.5 kb *Hind*III—*Bam*HI fragment from pNEO (P-L Biochemicals) containing the neomycin resistance gene. pSY1626 was cut and made blunt at the *Bam*HI site, and ligated with sheared ILTV fragments as described above. The ligation mixture was packaged using Gigapack XL (Stratagene) according to the manufacturers instructions. The packaging mixture was added to AG1 cells (Stratagene) grown in the presence of maltose, and colonies were selected on LB plates containing kanamycin. Cosmid subclones containing ILTV DNA were identified by comparing restriction enzyme maps of individual cosmid clones to each other and to ILVTV genomic DNA to obtain a contiguous sequence of ILTV

genomic DNA.

SCREEN FOR RECOMBINANT ILTV EXPRESSING ENZYMATIC MARKER GENES. When the *E. coli* β-galactosidase or β-glucuronidase (uidA) marker gene was incorporated into a recombinant virus the plaques containing the recombinants were visualized by a simple assay. The enzymatic substrate was incorporated (300 μg/ml) into the agarose overlay during the plaque assay. For the lacZ marker gene the substrate Bluogal™ (halogenated indolyl-β-D-galactosidase, Gibco BRL) was used. For the uidA marker gene the substrate X-Glucuro Chx (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid Cyclohexylammonium salt, Biosynth AG) was used. Plaques that expressed active marker enzyme turned blue. The blue plaques were then picked onto fresh cells and purified by further blue plaque isolation. In recombinant virus strategies in which the enzymatic marker gene was removed, the assay involves plaque purifying white plaques from a background of parental blue plaques. Viruses were typically purified with five to ten rounds of plaque purification.

SCREEN FOR FOREIGN GENE EXPRESSION IN RECOMBINANT ILTV USING BLACK PLAQUE ASSAYS. To analyze expression of foreign antigens expressed by recombinant ILT viruses, monolayers of CEK cells were infected with recombinant ILT virus, overlaid with nutrient agarose media and incubated for 3-5 days at 39°C. Once plaques have developed, the agarose overlay was removed from the dish, the monolayer rinsed once with PBS, fixed with 100% methanol for 10 minutes at room temperature and the cells air dried. After re-hydrating the plate with PBS, the primary antibody was diluted to the appropriate dilution with PBS plus Blotto and incubated with the cell monolayer for 2 hours to overnight at room temperature. Unbound antibody was removed from the cells by washing four times with PBS at room temperature. The appropriate secondary antibody conjugate was diluted 1:500 with PBS and incubated with the cells for 2 hours at room temperature. Unbound secondary antibody was removed by washing the cells three times with PBS at room temperature.The monolayer was rinsed in color development buffer (100mM

Tris pH 9.5/ 100mM NaCl/ 5mM MgCl2), and incubated 10 minutes to overnight at room temperature with freshly prepared substrate solution (0.3 mg/ml nitro blue tetrazolium + 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphatase in color development buffer).The reaction was stopped by replacing the substrate solution with TE (10mM Tris, pH7.5/ 1 mM EDTA). Plaques expressing the correct antigen stain black.

PURIFICATION OF ILTV gG FROM ILT VIRUS OR RECOMBINANT VIRUSES EXPRESSING ILTV gG. ILTV gG was purified from the media of cells infected with either wild type ILTV or with FPV or SPV vectors expressing ILTV gG. Cells were allowed to go to complete cytopathic effect (CPE), the media was poured off, and cell debris was pelleted in a table-top centrifuge. The media was concentrated in an Amicon concentrator using a YM30 ultrafiltration membrane at 15 psi. The concentrate was dialyzed against 20 mM Tris-HCl, pH 7.0 and loaded onto a DEAE-Sephacel (Pharmacia) column equilibrated with the same buffer. The material was eluted using a salt gradient from 0 to 1.5 M NaCl in 20 mM Tris-HCl, pH 7.0. Three ml fractions were collected and assayed by Western blot. A peptide antibody against ILTV gG was used to identify fractions containing ILTV gG. Fractions were pooled and further concentrated in a Centricon-10 microconcentrator (Amicon).

GROWTH OF CHICKEN KIDNEY CELLS AND ILT VIRUS. An ILTV virus, designated fowl laryngotracheitis challenge virus, lot number 83-2, was obtained from the National Veterinary Services Laboratories, USDA/APHIS, Ames, Iowa. ILTV viruses were grown in primary chicken kidney cells (CK) obtained by dissection of kidneys from 6-9 day old SPF chicks, obtained from Hy-Vac Laboratory Eggs Co. Fresh kidney cells were minced and disassociated with 5 mg/ml trypsin and were then pelleted and resuspended at 1.3 x 10^6 cells/ml. Growth media (GM) was 1X Eagle's Basal Medium (modified) with Hank's salts, with added 10% binary ethyleneimine-treated fetal bovine serum (FBS), 2 mM glutamine, 200 units/ml penicillin, 200 mg/ml streptomycin, and 8.9 mM sodium bicarbonate (85). After resuspension, cells were plated and

incubated at 39°C. Cells were rinsed and fed after 24 hours with maintenance media (MM), which is GM with 1% FBS. CKs were inoculated with ILTV at 0.01 to 0.1 MOI and viral stocks were harvested 4-5 days later by scraping and sonicating. Titers were typically 10^5-10^6 pfu/ml.

PREPARATION OF VIRAL DNA. Cells and media from infected flasks were pelleted at 1700 g for 5' at 4°C. Supernatant and cell pellet were initially treated separately. Virion particles were centrifuged out of the supernatant at 23,500 g for 30 minutes. The original cell pellet was rinsed with PBS and spun again. This pellet was resuspended in 1 ml/flask of a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 1.5 mM $MgCl_2$ and incubated 15', 4°C. To this was added 25 µl/flask of 20% NP40, and the mixture was dounce homogenized using an A pestle. The preparation was centrifuged at 1700 g, 10', 4°C, and the supernatant was retained and the pellet discarded. To the supernatant was added (per original flask) 10 µl of 0.5 M EDTA, 50 µl of 20% SDS, and 25 µl of 10 mg/ml proteinase K. This mixture was used to resuspend the pellet of viral particles obtained by high speed centrifugation of the first supernatant. The mixture was treated at 65°C for 1-16 hours, extracted twice with buffer-saturated phenol, and precipitated with added salt and ethanol. The resulting DNA pellet was resuspended in 100 µl TE/flask. This was treated further to remove RNA with pancreatic RNase A, followed by phenol extraction and ethanol precipitation to obtain the DNA.

CREATION OF THE COSMID LIBRARY. The cosmid library of ILTV DNA was created following the protocol of van Zijl *et al.*, (83). Approximately 20 µg of ILTV DNA in 0.5 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) was sheared by passing it twice through a 25 gauge needle. The DNA was centrifuged through a 15-40% glycerol gradient in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl for 5.5 h at 274,000 g. Fractions were analyzed on a 0.3% agarose gel, and those containing DNA of 35-50 kb were pooled, diluted twofold with TE, and precipitated with added salt and ethanol. The tubes were spun 1 h at 10°C and 109,000 g. Pellets were resuspended and

reprecipitated with added salt and ethanol. The DNA was resuspended in TE and the ends were made blunt by treatment with T4 DNA polymerase for 2 h at 15°C, in the presence of appropriate buffer and 25 μM dNTP, followed by treatment with Klenow polymerase for 16 h at 15°C using 0.25 mM dNTP. The DNA was extracted with phenol and then ether, precipitated with added salt and ethanol, and resuspended in TE. This material was ligated overnight with 3 μg of cosmid vector pSY1626. Cosmid pSY1626 was made by digesting cosmid pHC79 (BRL) with *Hin*dIII and *Ava*I to remove the tetracycline gene. The remaining fragment and the *Eco*RI digested polylinker from pWE15 (Stratagene) were filled in with Klenow polymerase and ligated together. The resulting cosmid vector, pSY1005, was modified at the *Eco*RI site to create pSY1626 by blunt-ended insertion of a 1.5 kb *Hin*dIII-*Bam*HI fragment from pNEO (P-L Biochemicals) containing the kanamycin resistance gene. PSY1626 was cut and made blunt at the *Bam*HI site for use as the cosmid vector. The ligation mixture was packaged using Gigapack XL (Stratagene) according to the manufacturer's directions. Colonies were selected on LB plates containing kanamycin.

SEQUENCING. Manual sequencing was performed using ^{35}S-dATP (NEN) with the BRL Sequenase Kit which uses the dideoxyribonucleotide chain termination method described by Sanger *et al.* (80). Reactions using both dGTP and dITP mixes were performed to clarify areas of compression. Alternatively, compressed areas were resolved on 8% acrylamide gels that were 40% in formamide. Automatic fluorescence sequencing was performed using an Applied Biosystems (ABI) 373A DNA Sequencer. Subclones were made to facilitate sequencing. Internal primers were synthesized on an ABI 392 DNA synthesizer. Sequence was obtained for both strands and was assembled using DNAstar software. Manipulation and comparison of sequences was performed with DNAstar programs, Superclone and Supersee programs from Coral Software. Comparisons with GenBank were performed at the NCBI using the BLAST network service (58).

HOMOLOGY VECTOR 501-94. The plasmid 501-94 was constructed for the purpose of deleting a portion of the thymidine kinase (TK) gene coding region from the ILT virus (28). It incorporates the HCMV IE promoter and a screenable marker, the *E. coli* lacZ gene, flanked by ILT virus DNA. The HCMV IE promoter–*E. coli* lacZ gene is inserted in the opposite transcriptional orientation to the ILTV TK gene. Upstream of the marker gene is an approximately 1087 base pair fragment of ILTV DNA which includes the first 77 amino acid codons of the ILTV TK gene. Downstream of the lacZ gene is an approximately 675 base pair fragment of ILTV DNA which includes 80 amino acid codons at the 3' end of the ILTV TK gene. When this plasmid is used according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS, it will replace the DNA coding for amino acids 78 to 285 of the ILTV TK gene with DNA coding for the lacZ gene. The lacZ marker gene is under the control of the human cytomegalovirus (HCMV) immediate early (IE) gene promoter and also contains the pseudorabies virus (PRV) gX gene polyadenylation signal at the 3' end of the gene. A detailed description of the plasmid is given in Figures 5A-5D. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (42, 43). The plasmid vector is derived from an approximately 3002 base pair *Hind*III fragment of pSP64/65 (Promega). Fragment 1 is an approximately 1087 base pair *Hind*III to *Bcl*I subfragment of the ILTV 2.4 kb *Hind*III fragment. Fragment 2 is an approximately 5017 base pair *Sal*I to *Sal*I fragment containing the HCMV IE promoter, β-galactosidase (lacZ) marker gene, and PRV gX polyadenylation signal (see Figures 5A-5D). Fragment 3 is an approximately 675 base pair *Bcl*I to *Hind*III subfragment of the ILTV 2.4 kb *Hind*III fragment.

HOMOLOGY VECTOR 544-55.12. The plasmid 544-55.12 was constructed for the purpose of deleting a portion of the US2 gene coding region from the ILT virus and inserting a foreign DNA. It incorporates a screenable marker, the *E. coli* uidA gene flanked by ILT virus DNA. The PRV gX promoter–*E. coli* uidA gene is inserted in the opposite transcriptional orientation to the ILTV

US2 gene. Upstream of the uidA gene is an approximately 2300 base pair fragment of ILTV DNA which includes 41 amino acid codons at the 3' end of the US2 gene (SEQ ID NO 2: aa. 188–229). Downstream of the uidA gene is an approximately 809 base pair fragment of ILTV DNA which includes 22 amino acid codons at the 5' end of the US2 gene (SEQ ID NO 2: aa. 1–22). When this plasmid is used according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS, it will replace the ILTV US2 DNA coding for amino acids 23 to 187 with DNA coding for the *E. coli* uidA gene. The uidA marker gene is under the control of the pseudorabies virus (PRV) gX promoter and also contains the herpes simplex virus type 1 thymidine kinase (HSV-1 TK) gene polyadenylation signal at the 3' end of the gene. A detailed description of the plasmid is given in Figures 6A-6D. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (42, 43). The plasmid vector is derived from an approximately 2958 base pair *Asp*718I restriction fragment of a pSP18/pSP19 fusion such that the multiple cloning site is *Eco*RI/*Sac*I/*Asp*718I/*Sac*I/*Eco*RI. Fragment 1 is an approximately 2300 base pair *Asp*718I to *Dra*I subfragment (SEQ ID NO 1: Nucl. 1–405) of the ILTV 2.5 kb *Asp*718I fragment. Fragment 2 is an approximately 3039 base pair *Xba*I fragment containing the PRV gX promoter, the *E. coli* uidA gene, and the HSV-1 TK polyadenylation site (See Figures 6A-6D). Fragment 3 is an approximately 809 base pair *Xba*I to *Asp*718I subfragment of the ILTV 1097 bp *Asp*718I fragment (SEQ ID NO 1: Nucl. 905–1714).

HOMOLOGY VECTOR 562-61.1F. The plasmid 562-61.1F was constructed for the purpose of deleting part of the gI gene from the ILT virus and inserting a foreign DNA. It incorporates a screenable marker, the *E. coli* uidA gene, flanked by ILT virus DNA. The PRV gX promoter-*E. coli* uidA gene is transcribed in the opposite direction to the ILTV gI gene promoter. The 983 base pair deletion begins 12 base pairs upstream of the translation initiation codon and deletes 324 of 363 amino acid codons at the 5' end of the ILTV gI gene (SEQ ID NO 11: aa. 325–363). When this plasmid is used according to

the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS, it will replace the DNA coding for the ILTV gI gene with DNA coding for the *E. coli* uidA gene. A detailed description of the plasmid is given in Figures 7A-7D. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (42, 43). The plasmid vector is derived from an approximately 2647 base pair *Asp*718I to *Hind*III fragment of pUC19. Fragment 1 is an approximately 1619 base pair *Asp*718I to *Xba*I subfragment of the ILTV 8.0 kb *Asp*718I fragment (SEQ ID NO 1: Nucl. 7556–9175). Fragment 2 is an approximately 691 base pair *Xba*I to *Xho*I fragment (SEQ ID NO 1: Nucl. 9175–9861) generated by the polymerase chain reaction (PCR). The template was the ILTV 8.0 kb *Asp*718I fragment. The upstream primer 92.09 (5'-CCTAGCACCCTTGTATCGCG-3'; SEQ ID NO. 55) sits down at a site 821 base pairs upstream of the ILTV gI gene and synthesizes DNA toward the 3' end of the gene. The downstream primer 92.11 (5'-CGCCTCGAGTCCCAATGAATAGGCATTGG-3'; SEQ ID NO. 56) sits down at a site 12 base pairs upstream of the translation start site of the ILTV gI gene and synthesizes DNA toward the 5' end of the gD gene. The product of the PCR reaction is 818 base pairs. This DNA fragment is digested with *Xba*I at the 5' end (a restriction enzyme site present in the ILTV DNA) and *Xho*I at the 3' end (a restriction enzyme site created in the PCR primer—see underlined sequence) to create an approximately 691 base pair *Xba*I to *Xho*I fragment. Fragment 3 is an approximately 3051 base pair *Sal*I fragment containing the PRV gX promoter, the uidA gene, and the HSV-1 TK polyadenylation site (See Figures 6A-6D). Fragment 4 is an approximately 624 base pair *Xho*I to *Hind*III fragment generated by PCR (SEQ ID NO 1: Nucl. 10,847–11,461). The template was the ILTV 8.0 kb *Asp*718I fragment. The upstream primer 92.10 (5'-CGCCTCGAGGACCCATGGTTGCGTGCG-3'; SEQ ID NO. 57) sits down at a site 117 base pairs upstream from the translation termination codon within the ILTV gI gene. The downstream primer 92.08 (5'-CTCGTCCGAACGAGTTACAG-3'; SEQ ID NO. 58) sits down at a site 604 base pairs downstream of the translation termination site of the ILTV gI gene and within the ILTV gE gene. The PCR product (729 base pairs) is digested

with *Xho*I which is a unique site generated by the upstream PCR primer (underlined) and with *Hind*III at a site within the ILTV gE gene. Restriction endonuclease digestion with *Xho*I and *Hind*III creates an approximately 624 base pair Fragment 4. Fragment 5 is an approximately 2700 base pair *Hind*III subfragment of the ILTV 8.0 kb *Asp*718I fragment (SEQ ID NO 1: Nucl. 11,461–13,473 plus unsequenced DNA).

HOMOLOGY VECTOR 472-73.27. The plasmid 472-73.27 was constructed for the purpose of deleting a portion of the glycoprotein G (gG) gene coding region from the ILT virus and inserting a foreign DNA. It incorporates a screenable marker, the *E. coli* lacZ gene, flanked by ILT virus DNA. The HCMV IE promoter-*E. coli* lacZ gene is transcribed in the same direction to the ILTV gG gene promoter. The 874 base pair deletion of the ILTV gG gene extends from 60 nucleotides upstream of the translation initiation site to 814 nucleotides into the amino acid coding sequence, removing the coding capacity of 271 of 292 amino acids of the gG protein (SEQ ID NO 7). When this plasmid is used according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS, it will replace the DNA coding for amino acids 1 to 271 of the ILTV gG gene with DNA coding for the *E. coli* lacZ gene. A detailed description of the plasmid is given in Figures 4A-4D. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (42, 43). The plasmid vector is derived from an approximately 2686 base pair *Asp*718I restriction fragment of pUC 19 (Gibco, BRL). Fragment 1 is an approximately 2830 base pair *Asp*718I to *Nhe*I subfragment of the ILTV 5164 bp *Asp*718I fragment (SEQ ID NO 1: Nucl. 1714–4544). Fragment 2 is an approximately 5017 base pair *Sal*I to *Sal*I fragment containing the HCMV IE promoter, *E. coli* β-galactosidase (lacZ) marker gene, and PRV gX polyadenylation signal (see Figures 4A-4D). Fragment 3 is an approximately 1709 base pair *Sal*I to *Asp*718I subfragment of the ILTV 5164 bp *Asp*718I fragment (SEQ ID NO 1: Nucl. 5419–6878).

HOMOLOGY VECTOR 560-52.F1. The plasmid 560-52.F1 was constructed

for the purpose of deleting part of the UL47-like gene, all of ORF4, and part of the ILTV gG gene from the ILT virus and inserting a foreign DNA. It incorporates a screenable marker, the *E. coli* uidA gene, flanked by ILT virus DNA. The PRV gX promoter-*E. coli* uidA gene is transcribed in the opposite direction to the ILTV UL47-like, ORF4, and gG gene promoters. The 2640 base pair deletion removes 442 of 511 amino acid codons at the 3' end of the UL47-like gene (SEQ ID NO 4), the entire coding sequence of the ORF4 gene (SEQ ID NO 5) and 271 of 293 amino acid codons at the 5' end of the ILTV gG gene (SEQ ID NO 7). When this plasmid is used according to the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS, it will replace the DNA coding for the ILTV UL47-like, ORF4 and gG genes with DNA coding for the PRV gX promoter–*E. coli* uidA gene. A detailed description of the plasmid is given in Figures 8A-8D. It was constructed from the indicated DNA sources utilizing standard recombinant DNA techniques (42, 43). The plasmid vector is derived from an approximately 2958 base pair *Asp*718I restriction fragment of pSP18/pSP19 such that the multiple cloning site is *Eco*RI/*Sac*I/*Asp*718I/*Sac*I/*Eco*RI. Fragment 1 is an approximately 1066 base pair *Asp*718I to *BssH*II subfragment of the ILTV 5164 bp *Asp*718I fragment (SEQ ID NO 1: Nucl. 1714–2777). Fragment 2 is an approximately 123 base pair *Sal*I to *Bcl*I subfragment of the ILTV 5164 bp *Asp*718I fragment. Fragment 3 is an approximately 3027 base pair *Bam*HI fragment containing the PRV gX promoter, the uidA gene, and the HSV-1 TK polyadenylation site (See Figures 8A-8D). Fragment 4 is an approximately 1334 base pair *Bcl*I to *Asp*718I subfragment of the ILTV 5164 bp *Asp*718I fragment (SEQ ID NO 1: Nucl. 5544–6878).

HOMOLOGY VECTOR 579-14.G2. The plasmid 579-14.G2 was constructed for the purpose of deleting the entire gG gene and a portion of the g60 gene from the ILT virus and inserting a foreign DNA. It incorporates a PRV gX promoter and a screenable marker, the *E. coli* uidA gene, flanked by ILT virus DNA. The PRV gX promoter-*E. coli* uidA gene is transcribed in the same direction to the ILTV gG and g60 gene promoters. The 3351 base pair deletion

*Bam*HI restriction fragment Q (48). Note that this fragment is oriented such that the polyadenylation sequence (AATAAA) is located closest to the junction with the *E. coli* uidA gene.

PLASMID 388-65.2. Plasmid 388-65.2 contains the β-galactosidase expression cassette consisting of the HCMV immediate early (IE) promoter, the *E. coli* lacZ marker gene, and the PRV gX gene polyadenylation site. A detailed description of the β-galactosidase expression cassette is given in Figures 11A-11D. It was constructed utilizing standard recombinant DNA techniques (42, 43) by joining restriction fragments from the following sources with the synthetic DNA sequences indicated in Figures 11A-11D. The plasmid vector pSP72 (Promega) is derived from an approximately 3076 base pair *Pst*I to *Pst*I fragment. Fragment 1 is a 1154 base pair *Pst*I to *Ava*II fragment derived from a HCMV 2.1 kb *Pst*I fragment containing the HCMV IE promoter. Fragment 2 is a 3010 base pair *Bam*HI to *Pvu*II fragment derived from plasmid pJF751 (49) containing the *E. coli* lacZ gene. Fragment 3 is an approximately 750 base pair *Nde*I to *Sal*I fragment derived from PRV BamHI #7 which contains the carboxy-terminal 19 amino acids and the polyadenylation signal of the PRV gX gene.

EXAMPLES

Example 1

Complete sequence of the unique short region of Infectious Laryngotracheitis Virus (ILTV): The sequence of 13,473 base pairs of contiguous DNA from the short region of the ILT virus (SEQ. ID. NO. 1) was determined. This sequence contains the entire 13,098 base pair unique short region as well as 273 base pairs of repeat region at one end and 102 base pairs of repeat region at the other end. The unique short region contains 13 methionine initiated open reading frames (ORF) of greater than or equal to 110 amino acids (excluding smaller nested ORFs). All 13 ORFs were aligned to the Entrez release 6.0 virus division of the Genbank DNA database utilizing the IBI MacVector Protein to DNA alignment option (default settings). Eight of the ORFs exhibited significant homology to one or more other virus genes (see Table I). The nucleotide sequence numbers referred to below begin within the internal repeat sequence and end within the terminal repeat sequence. The unique short region begins at base pair 274 of SEQUENCE ID NO. 1.

Table I

Sequence Homology between Infectious Laryngotracheitis Virus (ILTV) Open Reading Frames in the Unique Short Region and other Viral Proteins

Open Reading Frame(ORF)	Start(BP)	End(BP)	Length(aa)	Genbank Allignmenta
1 (Rc)[b]	970	281	229	EHV-1 US2
2	1059	2489	476	MDV PK
3	2575	4107	510	HSV-1 UL47
4	4113	4445	110	NSc
4 (RC)	4519	4139	126	NS
5	4609	5487	292	PRV gX
6	5697	8654	985	ILTV g60
6 (RC)	7826	6948	292	HSV-2 UL39
7	8462	9766	434	PRV g50
8	9874	10962	362	VZV gI
8 (RC)	11150	10617	177	NS
9	11159	12658	499	VZV gE
10	12665	13447	260	NS

[a] Sequence allignment scored to the Entrez Release 6.0 of Genbank Virus Database.
[b] RC=Reverse Complement.
[c] NS=No score above 120 was found.
Other Abbreviations: EHV= Equine herpesvirus; MDV= Mareks disease virus; HSV-1= Herpes Simplex virus 1; PRV= Pseudorabies virus; ILTV= Infectious laryngotracheitis virus; HSV-2= Herpes Simplex virus 2; VZV= Varicella–Zoster virus; BP= base pairs; aa= amino acids.

US2 gene

The US2 gene consists of 690 base pairs and codes for a protein 229 amino acids in length and molecular weight approximately 25,272 daltons (SEQ. ID. NO. 12, 13). The ILTV US2 is homologous to the Equine herpesvirus(EHV)-1 and EHV-4 US2 proteins. The US2 gene is transcribed from nucleotide 970 to

281 on the reverse complement strand of the ILTV unique short region (SEQ. ID. NO. 1). The function of the US2 gene product is unknown.

Protein kinase gene

The protein kinase gene consists of 1431 base pairs from nucleotide 1059 to 2489 and codes for a protein 476 amino acids in length and molecular weight approximately 54,316 daltons (SEQ. ID. NO. 2). The ILTV protein kinase is homologous to the protein kinases from Mareks disease virus (MDV), Equine herpesvirus(EHV)–1 and –4, Pseudorabies virus (PRV), Varicella–Zoster virus (VZV), Simian varicella virus (SVV), and Herpes Simplex virus(HSV)–1 and –2.

UL47–like gene

The UL47–like gene is unique in its location within the unique short region of ILT virus. The UL47–like gene in all other known herpesviruses is located within the unique long sequence. The UL47–like gene consists of 1533 base pairs from nucleotide 2575 to 4107 and codes for a protein 510 amino acids in length and molecular weight approximately 57,615 daltons (SEQ. ID. NO. 3).

ORF4

ORF4 codes for a protein of unknown function. ORF4 consists of 333 base pairs from nucleotide 4113 to 4445 and codes for an open reading frame 110 amino acids in length and molecular weight approximately 12,015 daltons (SEQ. ID. NO. 4).

ORF4 Reverse Complement

ORF4 Reverse Complement (RC) codes for a protein of unknown function. ORF4 RC consists of 380 base pairs from nucleotide 4519 to 4139 and codes for an open reading frame 126 amino acids in length and molecular weight

approximately 13,860 daltons (SEQ. ID. NOS. 14, 15).

gG gene

The gG gene consists of 879 base pairs from nucleotide 4609 to 5487 and codes for a glycoprotein 292 amino acids in length and molecular weight approximately 31,699 daltons (SEQ. ID. NO. 5). ILTV gG glycoprotein is homologous to PRV gX, Bovine herpesvirus(BHV)-1.3 gG, EHV-1 gG and EHV-4 gG. Recombinant ILTV gG protein produced in a Swinepox virus vector or a Fowlpox virus vector can be purified (see Materials and Methods) and reacts to peptide antisera to ILTV gG. The peptide antisera reacts to ILTV gG from wild type virus, but not to viruses deleted for the ILTV gG gene. Deletion of the gG gene results in an attenuated ILT virus that is useful as a vaccine against ILT disease in chickens (see table in Example 6) and also serves as a negative marker to distinguish vaccinated from infected animals.

g60 gene

The g60 gene has been identified as glycoprotein 60 (33, 53). The g60 gene consists of 2958 base pairs from nucleotide 5697 to 8654 and codes for a glycoprotein 985 amino acids in length and molecular weight approximately 106,505 daltons (SEQ. ID. NO. 6).

ORF6 Reverse Complement

ORF6 RC consists of 878 base pairs from nucleotide 7826 to 6948 and codes for an open reading frame 292 amino acids in length and molecular weight approximately 32,120 daltons (SEQ. ID. NO. 16, 17). The ILTV ORF6 RC shares limited homology to portions of the HSV-1 and HSV-2 ribonucleotide reductase large subunit (UL39).

gD gene

The expression of the gD glycoprotein in vectored fowlpox virus or herpesvirus of turkeys (33) is sufficient to raise a protective immune response in the chicken. The gD gene consists of 1305 base pairs from nucleotide 8462 to 9766 and codes for a glycoprotein 434 amino acids in length and molecular weight approximately 48,477 daltons (SEQ. ID. NO. 10, 11). The ILTV gD glycoprotein is homologous to the PRV g50, and the gD from HSV-1, MDV, IPV, and BHV-1.1. Monoclonal antibodies raised to ILT virus react specifically with gD protein from ILTV and also react to ILTV gD protein expressed in a Herpesvirus of Turkeys (HVT) virus vector. ILTV gD expressed in the HVT vector is useful as a subunit vaccine.

gI gene

The gI gene consists of 1089 base pairs from nucleotide 9874 to 10,962 and codes for a glycoprotein 362 amino acids in length and molecular weight approximately 39,753 daltons (SEQ. ID. NO. 7). The ILTV gI glycoprotein is homologous to the VZV gI. Recombinant ILTV gI protein expressed in a swinepox virus vector reacts to convalescent sera from ILTV-infected chickens. Deletion of the gI gene results in an attenuated ILT virus that is useful as a vaccine against ILT disease in chickens. Recombinant viruses deleted for gI are safe in animal trials when vaccinated by a natural route directly into the respiratory tract, whereas parental virus causes lesions in 90% of the birds inoculated via the same route. Deletion of the gI gene serves as a negative marker to distinguish vaccinated from infected animals.

ORF8 Reverse Complement

ORF8 Reverse Complement codes for a protein of unknown function. ORF8 RC consists of 533 base pairs from nucleotide 11,150 to 10,617 and codes for an open reading frame 177 amino acids in length and molecular weight approximately 19,470 daltons (SEQ. ID. NO. 18, 19).

<u>gE gene</u>

The gE gene consists of 1500 base pairs from nucleotide 11,159 to 12,658 and codes for a glycoprotein 499 amino acids in length and molecular weight approximately 55,397 daltons (SEQ. ID. NO. 8). The ILTV gE glycoprotein is homologous to the gE glycoproteins from VZV, Simian herpesvirus (SHV), EHV-1, HSV-1, and PRV. The ILTV gE is a neutralizing antigen useful as a subunit vaccine.

<u>ORF10</u>

ORF10 consists of 783 base pairs from nucleotide 12,665 to 13,447 and codes for a protein 261 amino acids in length and molecular weight approximately 27,898 daltons (SEQ. ID. NO. 9).

Example 2

S-ILT-004

S-ILT-004 is an infectious laryngotracheitis virus (ILTV) that has an approximately 620 base pair deletion of the thymidine kinase (TK) gene (28). The gene for *E. coli* β-galactosidase (lacZ) was inserted in the place of the TK gene and is under the control of the HCMV immediate early (IE) promoter. Transcription of the HCMV IE promoter–lac Z gene is in the opposite orientation to the TK promoter.

S-ILT-004 was constructed using homology vector 501-94 (see Materials and Methods) and S-ILT-001 (USDA ILTV Strain 83–2) in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock was screened by the Bluogal™ SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was recombinant virus S-ILT-004. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING OF DNA procedure. This analysis confirmed the presence of the β-galactosidase (lacZ) marker gene and the deletion of approximately 619 base pairs of the TK gene. The remaining TK gene sequence codes for protein including amino acids 1 to 77, and amino acids 286 to 363. The HCMV IE promoter-lacZ gene is in the opposite orientation to the TK gene transcription.

S-ILT-004 is attenuated by deletion of the ILTV TK gene, but retains other genes known to be involved in the immune response in chickens to ILT virus. Therefore, S-ILT-004 may be useful as a killed vaccine to protect chickens from ILT disease.

Example 3

S-ILT-009

S-ILT-009 is an infectious laryngotracheitis virus (ILTV) that has an approximately 498 base pair deletion of the ILTV US2 gene and an approximately 874 base pair deletion of the ILTV gG gene. The gene for *E. coli* β-glucuronidase (uidA) was inserted in the place of the US2 gene and is under the control of the pseudorabies virus (PRV) gX promoter.

S-ILT-009 was constructed using homology vector 544-55.12 (see Materials and Methods) and S-ILT-002 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. S-ILT-002 was constructed as described in Example 5 (S-ILT-014). The transfection stock was screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The resulting purification of a blue plaque was recombinant virus S-ILT-009. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING OF DNA procedure. This analysis confirmed the presence of the PRV gX promoter–β-glucuronidase (uidA) marker gene and the deletion of approximately 498 base pairs of the ILTV US2 gene and an approximately 874 base pair deletion of the ILTV gG gene. However, during the Bluogal™ SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES, a deletion of the HCMV IE promoter-lacZ gene was detected within the existing ILTV gG deletion. The remaining insert into the ILTV gG deletion contains approximately 2000 base pairs of DNA of which all of the lacZ gene and part of the PRV gX polyadenylation site are missing. The deletion was characterized by detailed restriction mapping and determined to be slightly different from the S-ILT-014 deletion (See Example 5).

S-ILT-009 is attenuated by deletion of the ILTV US2 and gG genes, but retains other genes known to be involved in the immune response in chickens to ILT

virus. Therefore, S-ILT-009 is useful as an attenuated live vaccine or as a killed vaccine to protect chickens from ILT disease as shown in the table. Since S-ILT-009 does not express the ILTV gG genes, it is utilized as a negative marker to distinguish vaccinated animals from infected animals as described previously.

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Table II

EFFICACY OF RECOMBINANT LIVE ILT VIRUS S-ILT-009 AGAINST VIRULENT INFECTIOUS LARYNGOTRACHEITIS VIRUS CHALLENGE

Vaccine	**Gene(s) Deleted**	**Dose**	**Route**	**Challenge**[a]	**Protection**[b]
S-ILT-009	gG-, US2-	$7.8x10^3$	IO [c]	OS [d]	70%
S-ILT-009	gG-, US2-	$1.56x10^3$	IO	OS	77%
Controls				OS	0%
ASL embryo			IO	OS	90%

14 day old chicks
a: USDA Challenge virus $=1.0x10^{4.5}$ pfu
b: Protection = # healthy birds/total (%).
c: Intraocular
d: Orbital Sinus

Example 4

S-ILT-011

S-ILT-011 is an infectious laryngotracheitis virus (ILTV) that has an approximately 983 base pair deletion of the ILTV gI gene. The gene for *E. coli* β-glucuronidase (uidA) was inserted in the place of the gI gene and is under the control of the pseudorabies virus (PRV) gX promoter. The PRV gX promoter–uidA gene is in the opposite orientation to the direction of transcription of the ILTV gI promoter.

S-ILT-011 was constructed using homology vector 562-61.1F (see Materials and Methods) and S-ILT-001 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock was screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was recombinant virus S-ILT-011. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING OF DNA procedure. This analysis confirmed the presence of the β-glucuronidase (uidA) marker gene and the deletion of approximately 983 base pairs of the ILTV gI gene which deletes 325 of 363 amino acid codons from the 5' end of the gI gene.

S-ILT-011 is attenuated and is useful as a killed vaccine to protect chickens from ILT disease. S-ILT-011 shows a small plaque phenotype in tissue culture which is indicative of slow viral growth and attenuation. Since S-ILT-011 does not express the ILTV gI gene, it may be utilized as a negative marker to distinguish vaccinated animals from infected animals. As indicated in Example 1, ILTV–infected chickens make antibodies against ILTV gI protein.

Example 5

S-ILT-013

S-ILT-013 is an infectious laryngotracheitis virus (ILTV) that has an approximately 983 base pair deletion of the ILTV gI gene and an approximately 874 base pair deletion of the ILTV gG gene (and a deletion of the HCMV IE promoter lacZ marker gene making the lacZ gene nonfunctional). The gene for *E. coli* β-glucuronidase (uidA) was inserted in the place of the gI gene and is under the control of the pseudorabies virus (PRV) gX promoter.

S-ILT-013 was constructed using homology vector 562-61.1F (see Materials and Methods) and S-ILT-014 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock was screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was recombinant virus S-ILT-013. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING OF DNA procedure. This analysis confirmed the presence of the β-glucuronidase (uidA) marker gene and the deletion of approximately 983 base pairs of the ILTV gI gene which removes 325 of 363 amino acid codons from the 5' end of the gI gene. This analysis also confirmed an approximately 874 base pair deletion of the ILTV gG gene and an approximately 1906 base pair insertion of a partial HCMV IE promoter–lacZ marker gene DNA, of which a portion of the HCMV IE promoter and almost none of the lacZ gene remains (see Example 6).

S-ILT-013 is attenuated and is useful as a killed vaccine to protect chickens from ILT disease. S-ILT-013 shows a small plaque phenotype in tissue culture which is indicative of slow viral growth and attenuation. Since S-ILT-013 does not express the ILTV gI or gG genes, ILTV gI and gG may be utilized as negative markers to distinguish vaccinated animals from infected animals.

Example 6

S-ILT-014

S-ILT-014 is an infectious laryngotracheitis virus (ILTV) that has an approximately 874 base pair deletion of the ILTV gG gene and a deletion of the inserted HCMV IE promoter lacZ marker gene making the lacZ gene nonfunctional. S-ILT-014 was derived from a purified S-ILT-002 virus stock in which a deletion of the HCMV IE promoter lacZ marker gene occurred.

S-ILT-002 was constructed using homology vector 472-73.27 (See Materials and Methods) and S-ILT-001 in the DNA TRABSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The virus S-ILT-002 has a 874 base pair deletion within the ILTV gG gene and an insertion of the *E. coli* β-galactosidase (lacZ) gene in place of the ILTV gG gene. However, during the Bluogal™ SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES, a white plaque was picked which contained a deletion of the lacZ gene within the ILTV gG deletion.

This virus, S-ILT-014, was characterized by restriction mapping, DNA SEQUENCING and the SOUTHERN BLOTTING OF DNA procedure. This analysis confirmed the presence of an approximately 874 base pair deletion of the ILTV gG gene and approximately 1956 base pair insertion of a partial HCMV IE promoter lacZ marker gene DNA (2958 base pairs deleted). The remaining HCMV IE promoter lacZ marker gene DNA consists of an approximately 686 base pair DNA fragment of the approximately 1154 base pair HCMV IE promoter and an approximately 1270 base pair DNA fragment containing approximately 520 base pairs of the 3010 base pair β-galactosidase (lacZ) marker gene and all of the approximately 750 base pair PRV gX polyadenylation signal.

S-ILT-014 is useful as an attenuated live vaccine or as a killed vaccine to

protect chickens from ILT disease as indicated in the table below. Since S-ILT-014 does not express the ILTV gG gene and ILTV–infected chickens make antibodies to gG as indicated in Example 1, ILTV gG is utilized as a negative marker to distinguish vaccinated animals from infected animals.

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Table III

EFFICACY OF RECOMBINANT LIVE ILT VIRUS S-ILT-014 AGAINST VIRULENT INFECTIOUS LARYNGOTRACHEITIS VIRUS CHALLENGE

Vaccine	**Gene(s) Deleted**	**Dose**	**Route**	**Challenge[a]**	**Protection[b]**
S-ILT-014	gG-	$1.08x10^4$	IO [c]	OS [d]	97%
S-ILT-014	gG-	$2.16x10^3$	IO	OS	97%
Controls				OS	0%
ASL embryo			IO	OS	90%

14 day old chicks

a: USDA Challenge virus $=1.0x10^{4.5}$ pfu

b: Protection = # healthy birds/total (%).

c: Intraocular

d: Orbitual Sinus

Example 7

S-ILT-015

S-ILT-015 is an infectious laryngotracheitis virus (ILTV) that has an approximately 2640 base pair deletion of the UL47–like gene, the ORF4 gene. and ILTV gG gene. The gene for *E. coli* β-glucuronidase (uidA) was inserted in the place of the UL47–like, ORF4, and gG genes and is under the control of the pseudorabies virus (PRV) gX promoter. The PRV gX promoter–uidA gene is in the opposite orientation to the direction of transcription of the ILTV UL47–like, ORF4, and gG promoters.

S-ILT-015 was constructed using homology vector 560-52.F1 (see Materials and Methods) and S-ILT-001 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock was screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was recombinant virus S-ILT-015. This virus was characterized by restriction mapping and the SOUTHERN BLOTTING OF DNA procedure. These results confirmed the presence of a 2640 base pair deletion which includes 442 of a total 511 amino acid codons at the 3' end of the UL47–like gene, all of the ORF4 gene and 271 of 293 amino acid codons of the 5' end of the gG gene.

S-ILT-015 is useful as an attenuated live vaccine or as a killed vaccine to protect chickens from ILT disease as indicated in the table below. Since S-ILT-015 does not express the ILTV gG gene, ILTV gG is utilized as a negative marker to distinguish vaccinated animals from infected animals.

Table IV

EFFICACY OF RECOMBINANT LIVE ILT VIRUS S-ILT-015 AGAINST VIRULENT INFECTIOUS LARYNGOTRACHEITIS VIRUS CHALLENGE

Vaccine	**Gene(s) Deleted**	**Dose**	**Route**	**Challenge[a]**	**Protection[b]**
S-ILT-015	gG-, UL47-like	$1.0x10^5$	IO [c]	OS [d]	70%
Controls				OS	0%
ASL embryo			IO	OS	90%

14 day old chicks

a: USDA Challenge virus $=1.0x10^{4.5}$ pfu

b: Protection = # healthy birds/total (%).

c: Intraocular

d: Orbital Sinus

Example 8

S-ILT-017

S-ILT-017 is an infectious laryngotracheitis virus (ILTV) that has an approximately 3351 base pair deletion of the ILTV gG gene, ORF4 gene and the g60 gene. The gene for *E. coli* β-glucuronidase (uidA) was inserted in the place of the ILTV gG and g60 genes and is under the control of the pseudorabies virus (PRV) gX promoter.

S-ILT-017 was constructed using homology vector 579-14.G2 (see Materials and Methods) and S-ILT-001 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock was screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES. The result of blue plaque purification was recombinant virus S-ILT-017.

S-ILT-017 is attenuated by deletion of the ILTV g60 and gG genes, but retains other genes known to be involved in the immune response in chickens to ILT virus. Therefore, S-ILT-017 may be used as a killed vaccine to protect chickens from ILT disease. Since S-ILT-017 does not express the ILTV gG or g60 genes, it is used as a negative marker to distinguish vaccinated animals from infected animals.

Example 9

Recombinant infectious laryngotracheitis viruses that express infectious bronchitis virus (IBV) spike and matrix protein genes:

A homology vector is used to generate ILT viruses containing the IBV Arkansas spike protein gene. The recombinant ILT virus contains a deletion of one or more ILTV genes, including gG, US2, UL47–like, and ORF4, and the insertion of two foreign genes: the *E. coli* β-glucuronidase gene (uidA) and the IBV Arkansas spike protein gene. The uidA gene is under the control of the PRV gX promoter and the IBV Arkansas spike protein gene is under the control of the HCMV IE promoter.

To construct a homology vector containing the foreign genes inserted into the ILT virus, a DNA fragment containing the HCMV–IE promoter, the IBV Arkansas spike protein and the HSV-1 TK polyadenylation signal is inserted into a restriction enzyme site at the position of the deletion of the ILTV gG gene in the ILTV homology vector. A DNA fragment containing the PRV gX promoter and the *E. coli* β-glucuronidase (uidA) gene is inserted into a unique restriction enzyme site within the ILTV homology vector. A recombinant virus is constructed by combining the final homology vector containing the IBV Arkansas spike gene and the *E. coli* β-glucuronidase (uidA) gene and S-ILT-001 in the DNA TRANSFECTION FOR GENERATING RECOMBINANT ILT VIRUS. The transfection stock is screened by the X-Gluc SCREEN FOR RECOMBINANT HERPESVIRUS EXPRESSING ENZYMATIC MARKER GENES to detect the presence of the uidA gene and by the BLACK PLAQUE ASSAY FOR FOREIGN GENE EXPRESSION to detect the presence of the IBV Arkansas spike protein.

A similar strategy is used to construct recombinant ILT viruses carrying the IBV S1 protein from Arkansas, Massachusetts,or Connecticut serotypes, IBV matrix protein from Arkansas, Massachusetts, or Connecticut serotypes, and

IBV nucleocapsid from Arkansas, Massachusetts, or Connecticut serotypes. The strategy is also used to construct recombinant ILT viruses carrying the Newcastle Disease virus (NDV) HN and F genes and the Infectious Bursal Disease virus (IBDV) polyprotein or portions thereof. The strategy is also used to construct recombinant ILT viruses carrying the Mareks Disease virus (MDV) gA, gD, and gB genes.

Recombinant ILT virus carrying these antigens are valuable as a multivalent vaccine to protect chickens from diseases caused by ILTV and one or more of the viruses IBV, NDV, IBDV, or MDV. Since the ILTV vaccines described here do not express ILTV gG, it is useful as a negative marker to distinguish vaccinated animals from infected animals.

Example 10

Vaccines utilizing ILTV to express antigens from various disease causing microorganisms:

Antigens from the following microorganisms are utilized to develop poultry vaccines: Chick anemia agent, Avian encephalomyelitis virus, Avian reovirus, Avian paramyxoviruses, Avian influenza virus ,Avian adenovirus, Fowl pox virus, Avian coronavirus, Avian rotavirus, Salmonella spp., E coli., Pasteurella spp., Haemophilus spp., Chlamydia spp., Mycoplasma spp., Campylobacter spp., Bordetella spp., Poultry nematodes, cestodes, trematodes, Poultry mites/lice, Poultry protozoa (Eimeria spp., Histomonas spp., Trichomonas spp.).

Example 11

A Genomic Map of Infectious Laryngotracheitis Virus and the Sequence and Organization of Genes Present in the Unique Short Region

A cosmid library of the ILTV genome was created to facilitate restriction

endonuclease mapping. Forty-three overlapping cosmids were analyzed by digestion with *Asp*718I and *Not*I. *Asp*718I was known to cut the genome relatively infrequently (63), and it was found that *Not*I cut the genome less than ten times, which enabled cutting the vector away from the ILTV DNA insert. Comparison of these cosmid digests allowed the order of the *Asp*718I fragments covering 85% of the ILTV genome to be determined (Figure 12). On the long end of the genome, seven cosmids were identified which all contained a *Not*I site 0.9 kb from the end of the cloned insert; all other cosmid inserts had heterogeneous ends from shearing. This 0.9 kb fragment was used as a probe (P1 in Figure 12) to genomic ILTV digested with *Asp*718I, *Not*I, or *Bam*HI; the sizes of the genomic fragments that hybridized were identical to the size of the fragments excised from the cloned cosmid insert, indicating that the cloned insert extended all the way or very close to the end of the unique long. The 0.9 kb fragment did not hybridize to other bands in the ILTV digest, consistent with previous reports that this virus resembles PRV, and contains no long repeat (66). Once the cosmid clones were ordered, the restriction sites for a more frequent cutting enzyme, *Bam*HI, were mapped.

The resulting map indicated that the cosmid library did not include clones from the unique short portion of the genome. Cosmids spanning the unique short region of HVT (76) and PRV (83) have been found to be underrepresented in cosmid libraries. The *Asp*718I fragments found in the cosmid clones with an *Asp*718I digest of wild type ILTV and identified fragments of 8.0, 5.1, and 2.5 kb which were not represented in the cosmid library (Figure 13) were compared. These fragments were cloned into plasmids and hybridized to each other and to ILTV digested with *Bam*HI. The *Asp*718I 2.5 and 8.0 kb fragments cross-hybridized, indicating that they contained sequence repeated in both clones. Fine mapping of the *Asp*718I 2.5 and 8.0 kb fragments showed them to contain 2.1 kb of identical sequence. Hybridization to ILTV digested with *Bam*HI identified *Bam*HI bands of 7.5, 6.5, and 4.5 kb which overlapped the *Asp*718I fragments. These *Bam*HI fragments were cloned and analyzed by restriction digestion and hybridization. This allowed the map of the entire

unique short region and some of the flanking short repeat to be elucidated (Figure 13). Subclones of this region were made, and the entire unique short region was sequenced.

To complete the genomic map, the map searched for an *Asp*718I or *Bam*HI fragment that spanned the region between the short repeat sequences of the 8.0 or 2.5 kb *Asp*718I fragments mentioned above and the unique long region identified in the cosmid map. A 10 kb *Not*I fragment from the rightmost end of cosmid D5 (Figure 12) was hybridized to genomic ILTV digests on Southern blots. Interestingly, ladders of hybridizing bands were seen when the enzymes *Bam*HI, *Not*I, and *Asp*718I were used. The bands corresponding to these ladders were not generally visible in ethidium bromide stained gels. Subsequent subcloning and mapping of the 10 kb D5 fragment indicated that it contained up to 5 repeats of an 856 bp segment, and that the cosmid insert ended within a repeat motif. *Hin*dIII, which cuts once within the repeat, was used to clone the 856 bp fragment. When this fragment (Figure 12, P2) was used to probe ILTV digested with *Sfi*I, *Not*I, *Asp*718I, and *Bam*HI, ladders of hybridization were again seen (Figure 14). These ladders arise from varying numbers of the 856 bp repeat in different viral molecules. *Sfi*I cuts only once in this ILTV strain, and a ladder at very high molecular weight can be seen. Because the unique short is expected to invert, two overlapping *Sfi*I ladders containing the unique short and terminal repeat (TR_s) should be present.; however, the bands are too large in this region to make this distinction. *Not*I and *Asp*718I cut further away from the repeat, generating ladders beginning at 10.5 or 12 kb. The *Asp*718I digest should generate two overlapping ladders, because one fragment is bounded by an *Asp*718I site in the unique long, while the other is bounded by the end of the TR_s. In contrast, only one ladder should be generated by the *Not*I digest. Comparison of Figure 14 lane c (*Not*I) with lane d (*Asp*718I) does suggest that in lane d a second ladder is superimposed on the first, starting somewhat higher. *Bam*HI cuts close to the repeated region, and a ladder beginning at 3.4 kb is found. *Hin*dIII cuts within the repeat and generates a strongly hybridizing 856 bp band, as well as the two flanking

*Hind*III fragments of about 1.1 and 2.5 kb, which each contain a portion of the repeated sequence. The presence of this 856 bp repeat accounted for the occasional observation of very fine submolar bands in ethidium bromide-stained *Asp*718I digests. It also accounted for the lack, in ethidium bromide-stained gels, of a molar or half-molar quantity *Asp*718I or *Bam*HI band greater than 10 kb, which was expected to span this region based on analysis of the cosmid clones. Instead, because of the presence of the 856 bp repeat, this band exists as many submolar bands comprising the ladder. As can be seen in the *Bam*HI digest, there can be thirteen or more repeats of the region. Comparison of the repeat sequence to the sequence submitted to GenBank by Johnson *et al.* (67) indicated that it corresponded (99% identity) to nucleotides 1140 to 1996 of their sequence, which is a region just upstream of the ILTV ICP4 gene. The relationship of the repeat to the surrounding sequence is depicted in Figure 15. Restriction digests indicate that the region to the right of the repeat as shown is similar in the two strains; however, the position of the BamHI site indicated to the left of the repeat differs between them.

To identify the remainder of the short repeat from the 856 bp repetitive region to the *Bam*HI fragments used for sequencing the unique short, the 8.0 kb *Asp*718I fragment containing part of the short repeat was used as a probe to a second cosmid library of ILTV. One cosmid, clone 2F12, hybridized to the probe. Restriction endonuclease analysis of 2F12 and comparison to the cosmid map indicated that it was not a single contiguous cosmid, but was composed of two large non-contiguous fragments (see Figure 12). The break in the rightmost fragment was within a repeat of the 856 bp region. This fragment included at least two 856 bp repeats, and extended 4.6 kb through the remainder of the short repeat into the unique short.

To identify the end of the TR_s, the 6.6 kb *Not*I fragment spanning the unique long and the short internal repeat (IR_s) (P3 in Figure 2) was used as a probe. It was noted that a 2.9 kb *Not*I fragment seen in gels stained with ethidium bromide was not represented in the restriction endonuclease map, and

considered that it might represent the end of the TR_s. Hybridization of a *Not*I digest of ILTV with P3 indicated that this was indeed the case (Figure 16). The 2.9 kb *Not*I band hybridizes, as does the 6.6 kb band corresponding to the probe. In the *Bam*HI digest, the predicted 13 kb fragment containing a portion of the IR_s and a 3.5 kb fragment corresponding to the end of the TR_s are evident. In the *Asp*718I digest, an overlapping 2.7 kb fragment from the unique long hybridizes, and the high molecular weight ladder described previously was seen.

Sequencing of the ILTV unique short and flanking region identified nine open reading frames in the unique region and two (duplicated) in the repeat region as diagrammed in Figure 13 (SEQ UD NO:59). Comparison of the proteins encoded by these ORFs to the GenBank database (BLAST homology seacrh, National Center for Biological Information, NCBI) demonstrated identity for most of the potential proteins with other known herpesvirus gene products. Table V summarizes the closest homologies found for each gene and gives the probability scores for those homologies as generated by the search program. ORF2 (SEQ ID NO:63), the protein kinase (PK) gene (SEQ ID NO:63), is the most highly conserved of the ILTV ORFs to its herpes homologues. In contrast, the glycoprotein genes are less conserved. It should be noted that portions of the sequences of the ILTV protein kinase, gG, and ORF 5 genes have been published (69, 70 and 81); however, these genes were mapped to the unique long region. A description of each of the nine unique short genes and the two genes in the flanking short repeat follows.

The first open reading frame in the unique short encodes a 229 aa protein showing identity to other herpesvirus US2 proteins (SEQ ID NO:62). Like other US2 genes, it is in the opposite orientation to the remaining ORFs in the unique short. The coding sequence of the gene ends just within the unique short region, and a potential poly-A addition site is found 115 bases downstream in the short repeat. Two possible TATA promoters are found 37 and 70 bases upstream from the initiation codon.

ORF2 encodes a protein kinase with strong identity to many other herpesvirus protein kinases and to cellular protein kinases . The organization of the US2 and PK genes, with their 5' ends close together and their promoters possibly overlapping, is similar to that found in other herpesviruses. Two TATA sequences are present 14 and 49 bases upstream of the PK start codon, and two polyadenylation signals are found, one immediately after the stop codon, and one 50 bases downstream.

ORF3 encodes a 623 aa protein with similarity to the herpes simplex virus UL47 gene (SEQ ID NO:64). The program comparing this protein with other UL47 proteins projects a poor probability score for this homology. However, at least one of the regions of identity between ILTV and HSV UL47 corresponds to a region that is conserved among other herpesvirus UL47 homologues, suggesting that this identity is significant (Figure 17). Additionally, it should be noted that equally poor probability scores for homology generated by comparisons of the gG or gI genes are also seen for certain homologue pairings, suggesting that these scores are not sufficient for determining homology. It is interesting that the ILTV UL47 gene, normally found in the unique long region of other herpesviruses, appears to have been transposed into the unique short in ILTV.

The fourth open reading frame encodes a 292 aa glycoprotein homologous to PRV gG (SEQ ID NO:65). Four N-linked glycosylation sites with the consensus sequence NXT or NXS are present. The protein has a signal sequence of 26 aa, which could be cleaved at G/AP, but lacks a transmembrane anchor. It is therefore likely that this protein is secreted, similar to other herpesvirus gG homologues. This gene has a consensus TATA sequence 83 bases upstream from the ATG start, and has two potential polyadenylation sites 73 and 166 bases downstream from the stop codon.

ORF5 could encode a protein of 985 amino acids (SEQ ID NO:66). A

hydrophobic signal sequence is found at the amino terminus, and a hydrophobic sequence is present at the carboxy terminus. Nine glycosylation sites are found, suggesting that this is a glycoprotein. ORF 5 contains an imperfect repeat, consisting of 30 to 36 bp repeated approximately 23 times from amino acid 431 to amino acid 677. The hydrophilic amino acid consensus sequence created by this repeat is FTQTPSTEPET/A. Comparison of ORF 5 with other herpesvirus sequences (Table V) shows similarity to the glycoprotein product from the equine herpesvirus 1 US5 gene (EUS5, 82). The low probability score for this identity arises primarily from the fact that both genes contain threonine-rich repeats. It is not clear whether this reflects homology in form, function, or both. Both the EUS5 and the ILTV ORF 5 genes are large, have similar positions among flanking genes in the unique short, have signal sequences, and encode glycoproteins, but other sequence similarities are not seen. It is interesting that the ORF 5 repeat region shows similarity to mucin genes, which also contain threonine rich repeats. The human mucin gene, for example, has the repeat GTQTPTTTPITTTTTVTPTPTPT, where 7 of the first 11 amino acids are identical to the ORF 5 repeat sequence. Again, whether this reflects a similarity in function of the encoded proteins is unclear. A TATA sequence is found 560 bases upstream of the start codon; the nearest consensus polyadenylation signal is at the end of the gI gene. This suggests that the ORF 5 transcript may be coterminal with the gD transcript.

The open reading frame for the gD homologue (ORF 6) (SEQ ID NO:67) overlaps the end of ORF 5. Four in-frame methionines are found within the first 58 amino acids of the open reading frame, and it is not clear which is the actual translational start codon. Because a potential TATA promoter sequence is located only 6-9 bases upstream from the first possible ATG codon, this codon would probably not be within RNA transcribed from this promoter; however, there are several TATA sequences further upstream that may also be used to initiate transcription. The other three potential initiation codons are found at aa 23, 47, and 58 within this ORF. Comparison of the sequences surrounding the four ATGs with the eukaryotic translational initiation consensus

sequence A/GCCATGG (71) suggests that the latter two ATG codons may be preferred translational start sites. The protein sequences derived from each of these starts were examined for the presence of eukaryotic signal sequences and signal cleavage sites. A start at aa 58 within the ORF would result in a signal peptide of 26 amino acids with a predicted cleavage site between two alanine residues. This same signal sequence would be positioned much further from the amino terminus and embedded in a more hydrophilic sequence if the other start sites were used. The start of ILTV gD was tentatively assigned to position 58. which would result in a protein 377 amino acids long. Of course, it is possible that more than one initiation codon is used *in vivo*. Experiments of Zelnik *et al.* (88) suggest that alternate in-frame ATG codons are used to initiate MDV and HVT gD transcription *in vitro*, though the *in vivo* situation was not addressed. Additional experiments on gD transcription and translation in ILTV are necessary to identify its translational start codon.

The ILTV gD homologue has a secretory signal sequence and a transmembrane helix (aa 352 - 372) at the carboxy terminus. Only one potential glycosylation site is found at position 250-252; this is of the form NPS, and may not be glycosylated due to the proline residue. There is some question, therefore, as to whether processed ILTV gD contains N-linked oligosaccharides. This would be similar to the gD homologue in pseudorabies virus, gp50, which also lacks N-linked glycosylation sites (75). As in other herpesviruses, the gD coding sequence lacks a poly-A addition signal immediately following the gene, and the closest signal is at the end of the gI gene.

The seventh open reading frame encodes a protein of 362 aa and is most homologous to varicella zoster virus glycoprotein I (SEQ ID NO:68). The encoded protein shows all the characteristics of related gI glycoproteins, including a signal sequence with a potential cleavage site at positions 22 and 23 between a glycine and an isoleucine, a transmembrane helix at the carboxy terminus from 272 - 292, and four possible N-linked glycosylation sites. A TATA sequence is present 51 bases upstream from the methionine start codon.

Two possible poly-A addition signals are found within the coding sequence for ILTV gI, and may be the signals used by the gD and ORF 5 transcription units upstream.

The gE gene (ORF 8) follows the gI. This gene is 499 aa long, and contains four N-linked glycosylation sites (SEQ ID NO:69). A signal sequence of 18 amino acids is present, and there are two and possibly three membrane-associated helices in the carboxy terminal portion of the protein. The gE gene has a TATA box 86 bases upstream of the start codon, and a potential poly-A addition signal just prior to the 3' end of the coding region. This may serve as the polyadenylation site for the gI gene.

The ninth open reading frame extends across the junction of the unique short and the short repeat, and could encode a protein of 260 amino acids (SEQ ID NO:70). This protein has no signal sequence or membrane anchor, but has one possible N-linked glycosylation site. In a search of GenBank, some similarity is found between this protein and BLRF2 of EBV, but the significance of this similarity is unknown. The poly-A addition signal in the short repeat may be utilized by this gene. A potential TATA sequence is found 178 bases upstream of the first ATG of this ORF.

The first open reading frame in the short repeat (SRORF1) (SEQ ID NOs: 61 and 71) encodes a 294 aa protein which displays homology to the gene product of MDV SORF3 (79 and 84) and HVT ORF3 (87). In MDV and HVT, the corresponding gene is found as one copy in the unique short, and its function is unknown. No homology has been identified with mammalian herpesviruses; this gene appears to be specific to avian herpesviruses. MDV SORF3 has been deleted by Parcells *et al.* (74), and does not appear to be absolutely required for infection in chickens.

SRORF2 encodes a protein of 278 amino acids with homology to other herpesvirus US10 genes (SEQ ID NOs:60 and 72). A zinc finger motif, found

in the EHV-4 US10, is highly conserved in the ILTV US10 (amino acids 201-218); this suggests that the ILTV US10 gene is a DNA binding protein. Regulatory sequences include a poly-A addition signal 163 bp after the stop codon; it is unclear where the promoter for this gene resides.

Discussion:

The organization of the genes in the unique short region of ILTV is similar to that seen in other herpesviruses. Several genes encoding glycoproteins are present, and the order of these genes is similar to that seen in equine herpesvirus 1, particularly with respect to ORF 5. Similarities to avian herpesviruses are also evident in the presence of the avian-specific gene, SRORF1, and its position relative to US2 and PK, though it differs from HVT and MDV in that it is in the short repeat and is duplicated, also appearing downstream from the ORF 9 gene. The PK gene itself has the most identity to MDV and HVT PK genes; however, other genes are found to be more like their homologues in diverse herpesviruses such as EHV, PRV, and SHV SA8. Unusual characteristics of the ILTV unique short are the inclusion of a gene normally found in the unique long, the UL47 homologue, and the presence of the unique gene, ORF 5, which contains a set of degenerate repeats.

This analysis of the structure of ILTV disagrees with previous reports. Comparison of the sequences described here with those of the Australian ILTV isolate SA-2 indicates that a 32 kd protein described by Kongsuwan *et al.* (70) is almost identical to the gG in this application, and the sequenced fragment of the g60 protein presented by Kongsuwan *et al.* (69) is part of the ORF 5 gene in this application. However, they identified the 5 kb *Asp*718I fragment containing both of these genes as coming from the unique long region of SA-2 (66). Recently, Guo *et al.* (62) reported the sequence of a region from the USDA challenge strain which they ascribed to the unique short on the basis of comparison to the map presented by Johnson *et al.* (66). No identity was found between this sequence and the unique short sequence described here. Instead,

the sequence described by Guo *et al.* (62) shows 98% identity to a sequence recently submitted to GenBank by Johnson *et al.* (67 and 68), which is reported to encode the ICP4 gene of ILTV. The *Bam*HI sites within the ICP4 coding region generate two contiguous fragments of 1.2 and 1.7 kb (see Figure 15). In the map described here, two contiguous *Bam*HI fragments of this size are found within the short repeats (Figure 12). In addition, the 856 bp repeat element, which is found just upstream of the ICP4 gene (Figure 15), was mapped in this application within the short repeats. This indicates that the ICP4 gene in the strain used in these studies is present in the IR_s and the TR_s. It is possible, but unlikely, that the Australian SA-2 vaccine strain underwent an unusual rearrangement which altered the relationship of the unique long, unique short, and short repeat. However, Guo *et al.* (62) used the same challenge strain as the one described in this application, and the sequence they reported is not in the unique short, but in the short repeats, similar to the ICP4 genes of other herpesviruses.

The gene encoded by ORF 5 contains threonine rich, degenerate repeats. These are similar in composition and in their repetitive nature to repeats found in mucin genes. This repeated region in mucin is modified by O-linked oligosaccharides and is highly hydrophilic. It is interesting to speculate on what the function of this somewhat similar region might be in infection, if it is expressed *in toto* in ILTV. At least a portion of this gene is known to be expressed, as Kongsuwan *et al.* (69) cloned and sequenced a fragment from it by probing a lambda gt11 library with a monoclonal antibody that was known to bind to a 60 kd ILTV protein (g60) on Western blots (86). The relationship of such a 60 kd protein to the predicted 985 aa product from ORF 5 is unknown. Comparison of the application sequence with the complete sequence of the g60 coding region (81) shows a 98.5% homology between the SA-2 strain and the USDA strain. Interestingly, there is an insertion of a block of 10 amino acids in g60 relative to the ORF 5 protein; this difference reflects one additional degenerate repeat sequence in the SA-2 strain.

As mentioned above, Kongsuwan *et al.* (70) described an ILTV gene that encoded a 32 kd protein with similarity to PRV gG. A comparison of the ILTV gG protein sequence described in this application with their 32 kd protein found 10 amino acid differences in the first 273 residues of the protein. At amino acid 274, a deletion of one base pair in SA-2 relative to the USDA strain created a frame shift, such that 19 additional residues were found in the challenge strain as opposed to 26 in SA-2. A peptide was made from the carboxy terminal sequence elicited antisera in mice which reacted with ILTV gG; this indicates that the sequence described in this application reflects the actual carboxy terminus in the USDA strain. A similar situation was found when the ILTV gD protein described in this application was compared with the ILTV gD sequence submitted to GenBank by Johnson *et al.* (68). Ten differences were found in the first 419 amino acids, after which a deletion of a base in the SA-2 strain relative to the the sequence described in this application caused the predicted carboxy termini to differ, with 15 more amino acids in the USDA strain and 9 in SA-2. These differences could arise from errors introduced during cloning and sequencing of these genes. It is also possible that the carboxy termini of the ILTV gG and gD genes are variable between these strains.

The 856 bp repeat unit identified within the short repeat is just upstream of the ICP4 gene described by Johnson *et al.* (67), but, from the sequence alone, it does not appear to be repetitive in the SA-2 strain. The *Bam*HI fragment containing this repetitive region is 2848 bp long in SA-2. The smallest repeat, seen faintly in the *Bam*HI ladder of Figure 14, is 3.4 kb long. This is not quite large enough to include two repeats, and suggests that other alterations between the two strains may exist in this region. A repeat of this sort has not been previously described for this or other ILTV strains, though the submolar nature of the bands may have obscured its presence. The appearance of the ladder is reminiscent of defective interfering particles, but it is not believed that this represents a case of defective interfering particles in the viral stock used here. Several reasons for this follow. 1) Defective interfering particles are generally

found when viruses are passaged at high multiplicity, and the ILTV viral stocks of this application were passaged at low multiplicity. In fact, viral stocks originating from a single picked plaque exhibited similar ladders when their DNA was subjected to Southern blot analysis, suggesting that a single viral particle containing a set number of repeats could regenerate the full range of the ladder after being grown for a short period of time. 2) If populations of defective interfering particles were present, one might expect to encounter digest fragments that would not be accommodated in the linear viral map (see, for example, 77), yet all but one of the cosmids analyzed make a contiguous map, with *Asp*718I bands identical to those present in genomic ILTV digests. The exception, 2F12, was unusual in being the only one of several hundred cosmid clones screened which contained part of the unique short. This probably represented an aberrant cloning event, and not a widespread phenomenon related to defective viral particles. 3) Defective interfering particles often are present in larger molar amounts than standard viral particles, such that restriction fragments originating from the defective particles are overrepresented. In contrast, the bands of the 856 bp ladder are submolar, and are only rarely visible in ethidium bromide stained gels. 4) Defective interfering particles contain origins of replication. The 856 bp repeat itself does not contain a herpesvirus origin of replication as defined by the consensus sequence of Baumann *et al.* (59). From these considerations it was concluded that varying numbers of 856 bp units are present in the short repeats of standard viral DNA from the USDA challenge strain of ILTV. Since fragments exist that contain thirteen or more repeats of the region, genomic DNA from ILTV could vary by over 11 kb in the short repeat regions. Repetitive regions have been identified in other herpesviruses; for example, Marek's disease virus contains a 132 bp repetitive sequence in the long repeat regions (61 and 73) and expansion of this repeat is associated with reduction of viral oncogenicity. The presence of the 856 bp tandem repeats in ILTV, in contrast, does not appear to affect viral pathogenicity, since this strain does cause severe clinical disease in chickens. It would be interesting to examine other ILTV strains for the presence of this repeat.

Table V indicates the ORFs of the ILTV unique short and the HSV nomenclature for these genes, in those cases where homology is found. The third column shows the best matches from the Blast homology search (NCBI), and the probability scores assigned by the program for the matches indicated. Smaller numbers indicate less likelihood that the match could occur randomly.

A genomic map of infectious laryngotracheitis virus (ILTV) and a 18,912 bp sequence containing the entire unique short region and a portion of the flanking short repeats is presented. In determining the genomic map, an 856 bp region repeated as many as 13 times was identified within the short repeats. The unique short sequence contains 9 potential open reading frames (ORFs). Six of these ORFs show homology to other known herpesvirus unique short genes. Using the herpes simplex virus nomenclature, these genes are the US2, protein kinase, and glycoproteins G, D, I, and E (SORFs 1, 2, 4, 6, 7, and 8, respectively). Interestingly, an open reading frame with homology to HSV-1 UL47 (SORF 3) is found in the unique short. One very large open reading frame (ORF 5) is present and contains a threonine rich, degenerate repeat sequence. This gene appears to be unique to ILTV among sequenced herpesviruses. Two ORFs were identified within the short repeat region. SRORF1 is homologous to a gene (SORF3) found in the unique short region in both MDV and HVT, and appears to be specific to avian herpesviruses. SRORF2 has homology to HSV US10.

Table V

ORF	HSV Homolog	Best Matches	Blast Score
1	US2	EHV1 EUS1	$3.1x10^{-13}$
		EHV4 EUS1	$5.3x10^{-12}$
		HSV2 US2	$6.7x10^{-7}$
2	PK	MDV PK	$8.2x10^{-36}$
		HVT PK	$5.4x10^{-35}$
		HSV1 PK	$4.1x10^{-30}$
3	UL47	HSV1 UL47	$6.0x10^{-1}$
		EHV1 UL47	$9.9x10^{-1}$
		MDV UL47	$9.9x10^{-1}$
4	gG	PRV gG	$5.3x10^{-5}$
		BHV1 gG	$1.7x10^{-2}$
		EHV1 gG	$6.8x10^{-1}$
5	ORF 5	EHV1 EUS5	$1.9x10^{-45}$
		Human mucin	$1.1x10^{-25}$

6	gD	MDV gD	$6.8x10^{-4}$
		PRV g50	$2.0x10^{-3}$
		HVT gD	$3.5x10^{-3}$
7	gI	VZV gI	$4.2x10^{-2}$
		HVT gI	$7.9x10^{-2}$
		SVV gI	$4.3x10^{-1}$
8	gE	SHV SA8 gE	$1.7x10^{-6}$
		HSV1 gE	$1.1x10^{-3}$
		BHV1 gE	$1.5x10^{-2}$
9	ORF 9	EBV BLRF2	$5.7x10^{-1}$
SR1	no HSV	MDV "ORF3"	$4.8x10^{-4}$
	homologue	HVT "ORF3"	$2.6x10^{-1}$
SR2	US 10	EHV-4 US10	$1.2x10^{-1}$
		HSV-1 US10	$8.7x10^{-1}$
		EHV-1 US10	$8.7x10^{-1}$

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